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The Generation of Antigen-Specific CD8+ T Cells from Stem Cells for Adoptive Transfer Immunotherapy

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The Generation of Antigen-Specific CD8+ T Cells from Stem Cells for Adoptive Transfer Immunotherapy

by

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Dedication

For my family

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The Generation of Antigen-Specific CD8+ T Cells from Stem Cells for Adoptive Transfer Immunotherapy

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Adoptive T cell transfer is a form of immunotherapy that has shown promise in treating several cancers and post-transplant lymphoproliferative diseases. This therapy relies on the unique ability of cytotoxic T lymphocytes to specifically recognize and eliminate pathogen-infected or malignant cells. Adoptive transfer involves the isolation of patient-derived T cells, followed by *ex vivo* expansion, and in some cases genetic manipulation, before infusion into the recipient. The procedure is often limited by the availability of donor cells, problems with primary cell expansion, the time required to generate adequate numbers of T cells, and the complications associated with using genetically modified cells *in vivo*. As a result, there is need for a high-throughput system from which large quantities of antigen-specific cytotoxic T cells can be generated.

The multipotency of stem cells makes them attractive, scalable cell sources for adoptive transfer T lymphocytes. In this work, functional, antigen-specific $CD8^+$ T cells were differentiated from human $CD34^+$ cord blood-derived hematopoietic stem cells, *in vitro*, using exogenous Notch ligands and peptide-loaded human leukocyte antigen tetramers. Tetramer-differentiated, progenitor-derived, antigen-specific $CD8^+$ T cells were then enriched and expanded using media supplemented with co-stimulatory

molecules and proliferative cytokines. The enriched T cells remained functional, but did not undergo robust expansion, suggesting that they entered a state of dysfunction. Lastly, the effects of peptide major histocompatibility complex (pMHC) density and surface presentation on thymocyte TCR signaling and antigen-specific differentiation were studied. Microplates and microbeads, fabricated with varying densities of pMHC molecules, were used to stimulate and differentiate thymocytes. Plate- and bead-immobilized pMHCs were more efficient at stimulating thymocytes compared to soluble pMHC tetramers, and were capable of inducing antigen-specific T cell differentiation in a density-dependent manner. In conclusion, the findings of this research indicate that antigen-specific $CD8^+$ T cells can be generated from progenitor cells *in vitro*, with the potential of high-throughput and large-scale production.

Table of Contents

List of Figures

Chapter 1: Overview, Specific Aims, and Outline

1.1 OVERVIEW

T lymphocytes are a unique set of white blood cells that play a critical role in the body's adaptive immune response. Over the past twenty years, scientists and clinicians have exploited the ability of $CD8^+$ cytotoxic T cells to specifically recognize and kill pathogen-infected or cancerous cells. This form of immunotherapy, known as adoptive T cell transfer, has shown promise in treating several cancers including metastatic melanoma, renal cancer, prostate cancer, B lymphoid malignancies, and nasopharyngeal cancer, as well as post-transplant lymphoproliferative diseases (PTLDs).[1-22] Adoptive transfer has conventionally involved the isolation of peripheral blood or tumor-infiltrating lymphocytes (TILs), followed by the enrichment and expansion of antigen-specific T cells *in vitro*.[23,24] Novel technologies to engineer cells with genes encoding exogenous T cell receptors (TCRs) or chimeric antigen receptors (CARs) have facilitated the generation of T cells with defined specificity, affinity, and avidity.[2,3,22,25]

Despite its immense promise, several limitations must be overcome in order for adoptive cell transfer to be translated into a widely used immunotherapy. The process of cell isolation from autologous or allogeneic donors is often difficult and inefficient. Populations of antigen-specific cytotoxic T lymphocytes (CTLs) or TILs may be very rare, making it difficult to obtain sufficient numbers during enrichment. In addition, it is not uncommon for TILs to be non- or sub-functional due to the immunosuppressive nature of the tumor microenvironment.[23,24] Expanding the desired T cell population to clinically relevant numbers ($> 10^{10}$ per infusion) can take several months - a time frame that is inappropriate for advanced diseases requiring immediate intervention.[23,24] While the use of T cells expressing exogenous receptors eliminates the need to screen and

enrich for a population of interest, it still relies on peripheral blood isolation and poses the potential of implanting genetically modified cells that could be oncogenic.

Therefore, a high-throughput *in vitro* system for generating antigen-specific T cells that can be used "on demand" is needed. The multipotency of stem cells, or their ability to differentiate into multiple types of cells, makes them attractive sources from which adoptive transfer T cells can be generated, and ideal candidates with which to study T lymphopoiesis. With the advent of umbilical cord blood (UCB) banks, hematopoietic stem cells (HSCs), which are capable of differentiating into the various blood lineages, have become readily accessible and routinely used for transplants in cancer patients. The retrieval of cord blood is relatively easy, as it is delivered during childbirth, and much work has already been done to optimize the isolation and expansion of cord blood-derived HSCs.[26]

T cells are unique from other subsets of white blood cells in that they develop within the thymus, not the bone marrow. In the adult, the start of T cell development begins when common lymphoid progenitors (CLPs) migrate into the thymus from the bone marrow. Two signals, presented by thymic epithelia are essential for proper T lymphopoiesis: (1) Notch receptor-Delta like ligand (DLL) signaling and (2) TCR-major histocompatibility complex (MHC) or human leukocyte antigen (HLA) signaling.[27,28] Work pioneered by Dr. Zúñiga-Pflücker's laboratory has shown that stromal cells expressing Delta like ligands can induce the development of mature T cells from mouse and human HSCs.[29-35] This system has been seminal for studying the biology of *in vitro* T cell differentiation; however it is not scalable or clinically relevant. Others have demonstrated that immobilized Notch ligands, with a defined combination of cytokines, can commit HSCs to the T lineage and induce early T cell development, although the

generation of mature, antigen-specific T cells using these stromal-free methods has not been shown.[36-40]

Antigen-specific T cells for adoptive transfer are typically obtained by expanding *ex vivo*-isolated T cells with epitope-loaded antigen-presenting cells (APCs).[23,24] Alternatively, numerous studies have shown that artificial antigen-presenting cell (aAPC) microbeads, functionalized with co-stimulatory molecules and/or peptide MHC (pMHC) or peptide HLA (pHLA) oligomers, can be used to expand specific T cell populations.[41-43] Based on work published by Dr. Krishnendu Roy's laboratory, showing that antigen-specific $CD8^+$ T cells can be generated from murine progenitors using pMHC Class I tetramers,[44] it was hypothesized that treating human stem cell-derived T cell progenitors with artificial pHLA tetramers could induce the differentiation of antigen-specific CTLs.

With this objective in mind, a scalable system with quantitatively controlled ligand presentation, free of stromal cell contact, was designed. This system, which employed immobilized Notch ligands, exogenous pHLA tetramers, and supporting growth factors, was used to differentiate $CD34^+CD38^{-/low}$ cord blood-derived hematopoietic progenitors into functional, antigen-specific $CD8^+$ T cells. In order for antigen-specific T cells to be used in the clinic, they must have the capacity to expand. In this dissertation, progenitor-derived antigen-specific $CD8^+$ T cells were enriched and cultured in the presence of T cell expansion factors. Results indicate that the enriched, progenitor-derived T cells remained functional throughout the expansion culture, but did not undergo robust proliferation. These studies, as well as possible solutions to enhance expansion, are described in detail in Chapter 4.

Within the thymus, the signaling strength resulting from TCR-pMHC engagement plays a critical role in positive and negative selection, as well as the CD4 versus CD8 lineage commitment, of developing T cells.[45-50] Many studies have focused on the role of

pMHC density and solid phase presentation on mature T cell activation and function;[51-55] however, much less is known about how these parameters influence the development of thymocytes *ex vivo* or how they affect antigen-specific differentiation. To investigate the effects of pMHC density and surface presentation on TCR signaling and T cell differentiation, T cell progenitors were cultured with microplates and beads functionalized with varying densities of pMHC molecules. It was found that pMHC-immobilized plates and beads could trigger TCR signaling more efficiently than soluble tetramers, and were capable of inducing antigen-specific differentiation in a density-dependent manner.

1.2 SPECIFIC AIMS

The specific aims of this exploratory and developmental research are as follows:

1.2.1 Aim 1: To generate functional, antigen-specific $CD8^+$ T cells from human $CD34^+$ umbilical cord blood hematopoietic progenitors using immobilized Notch ligands, soluble pHLA tetramers, and stromal cell-conditioned media:

In this aim, a system to generate antigen-specific $CD8^+$ T cells from $CD34^+$ UCB HSCs was rationally designed. Based on previous evidence, it was hypothesized that immobilized Notch ligands, in particular DLL1, could induce T lineage commitment and early T cell development. Secreted factors from the bone marrow-derived stromal cell line expressing DLL1, OP9-DL1, were included to support differentiation. To confirm successful T lineage commitment and early T cell development, the phenotype of the cells was evaluated after 16 and 25 days of culture with immobilized DLL1. At day 25, early T cells were cultured with pHLA Class I tetramers presenting cytomegalovirus (CMV) or influenza (GIL) epitopes. After 7 days, T cell maturation and antigen-specificity was confirmed using flow cytometry. The cytotoxic activity of the differentiated T cells against epitope-loaded target cells was determined by CD107a mobilization, interferon gamma (IFNγ) production, and granzyme B activity.

1.2.2 Aim 2: To enrich and expand progenitor-derived antigen-specific $CD8^+$ T cells to clinically relevant numbers:

Next, the capability of progenitor-derived antigen-specific $CD8^+$ T cells to expand to clinically relevant numbers was evaluated. Due to their easy accessibility and high cell yields, murine C57BL/6 double positive (DP) thymocytes were used as starting populations for these experiments. Sorted DP thymocytes were differentiated with pMHC tetramers presenting ovalbumin or lymphocytic choriomeningitis virus epitopes (OVA257p-MHC and LCMV.GP34p-MHC, respectively), in media supplemented with anti-CD3, anti-CD28, interleukin (IL)-2, and IL-7. After 7 days of differentiation,

antigen-specific $CD8^+$ single positive (SP) T cells were sorted and then expanded with the respective tetramers for an additional 7 days, in media containing anti-CD3, anti-CD28, and IL-2. After 7 days of expansion, cells were harvested and analyzed by flow cytometry to confirm antigen-specific enrichment. The functionality of the enriched, antigen-specific T cells was tested using granzyme B activity assays.

1.2.3 Aim 3: To quantitatively evaluate the effects of plate- and bead-immobilized pMHC oligomers on TCR signaling and the antigen-specific differentiation of immature thymocytes:

To complete this aim, microplates and microbeads were fabricated with OVA257p-MHC Class I molecules at varying densities. Ligand density was quantified, and DP thymocytes from OT-1 transgenic mice were cultured with OVA257p-MHC-functionalized microplates, microbeads, or soluble tetramers at equivalent numbers of pMHC molecules per cell. Ligand density was also varied to determine its effect on thymocyte stimulation. TCR signaling was evaluated by the expression of activation marker, CD69. To determine the efficiency of microplates and beads to induce antigen-specific differentiation, DP thymocytes from C57BL/6 mice were cultured with LCMV.GP34p-MHC-functionalized plates, beads, or soluble tetramers at equivalent numbers of pMHC molecules per cell. As with the signaling assays, ligand density was varied to determine its effect on thymocyte differentiation. T cell maturation and antigen-specificity were determined by flow cytometry.

1.3 OUTLINE

The body of this research dissertation focuses on generating functional, antigen-specific T cells from HSCs and progenitors, with the end goal of adoptive transfer immunotherapy. In the following chapter, **Chapter 2**, adoptive T cell transfer is outlined to provide the long-term motivation for this project. Information regarding *in vivo* and *in vitro* T cell development, from which the rational design of this project is based, is also discussed. In **Chapter 3**, the generation of human antigen-specific $CD8^+$ T cells from hematopoietic progenitors using a novel, scalable differentiation system, employing Notch and TCR ligands, is presented. The enrichment and expansion of progenitor-derived antigen-specific $CD8^+$ T cells is described in **Chapter 4**. **Chapter 5** details the fabrication of microplates and microbeads with varying densities of pMHC ligand. The ability of these surfaces to stimulate thymocytes and induce antigen-specific differentiation is investigated in **Chapter 5** as well. Lastly, **Chapter 6** discusses the implications of this work, as well as future directions of this research.

1.4 ABBREVIATIONS

PTLD – post-transplant lymphoproliferative disease

TIL – tumor-infiltrating lymphocyte

TCR – T cell receptor

CAR – chimeric antigen receptor

CTL – cytotoxic T lymphocyte

HSC – hematopoietic stem cell

UCB – umbilical cord blood

CLP – common lymphoid progenitor

DLL – Delta like ligand

MHC – major histocompatibility complex

HLA – human leukocyte antigen

APC – antigen-presenting cell

aAPC – artificial antigen-presenting cell

pMHC – peptide major histocompatibility complex

pHLA – peptide human leukocyte antigen

OP9-DL1 – stromal cells expressing Delta like ligand 1

CMV – cytomegalovirus

GIL – influenza

IFNγ – interferon gamma

DP – double positive

OVA257p-MHC – ovalbumin epitope (SIINFEKL) pMHC

LCMV.GP34p-MHC – lymphocytic choriomeningitis virus glycoprotein epitope (AVYNFATC) pMHC

IL – interleukin

SP – single positive

OT-1 – transgenic mouse line with T cells expressing receptors specific for OVA257p-MHC

SIINFEKL – (serine – isoleucine – isoleucine – asparagine – phenylalanine – glutamic acid – lysine – leucine)

AVYNFATC – (alanine – valine – tyrosine – asparagine – phenylalanine – alanine – threonine – cysteine)

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Chapter 2: Background and Significance

2.1 T CELL ADOPTIVE TRANSFER IMMUNOTHERAPY

Over the past two decades, the use of adoptive T cell transfer as a treatment for cancer and post-transplant lymphoproliferative diseases (PTLDs) has become a clinical reality due to advances in the fields of immunology and tumor biology.[1-23] Adoptive T cell therapy encompasses the use of circulating cytotoxic T lymphocytes (CTLs), tumor infiltrating lymphocytes (TILs), or genetically modified cells that express high-affinity/avidity T cell receptors (TCRs) or chimeric antigen receptors (CARs) as therapeutic agents (Figure 2.1).[17,18]

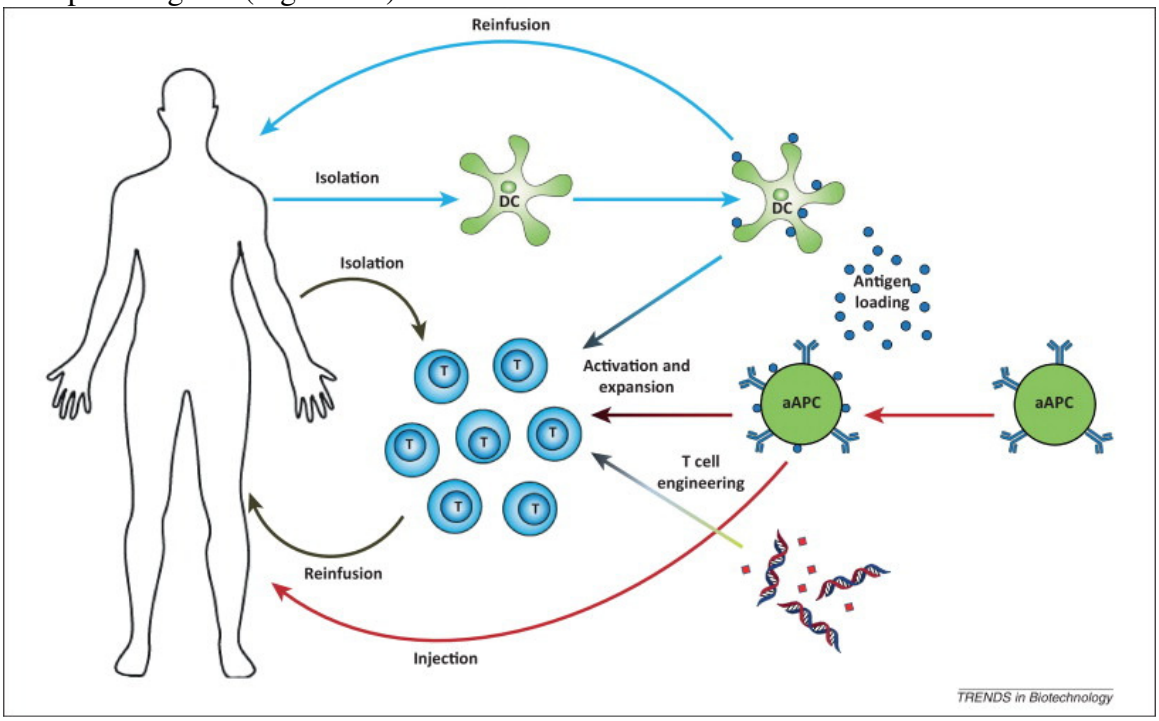

Figure 2.1: Schematic of adoptive transfer T cell therapy. Endogenous T cells (TILs or CTLs) are isolated, and expanded with antigen-presenting cells, or genetically modified with exogenous TCRs or CARs prior to reinfusion.*

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The efficacy of adoptive T cell transfer relies on the ability of T lymphocytes to specifically recognize and eliminate target cells with long-lasting immune protection. Numerous strategies have been developed to isolate and expand antigen-specific T cells, engineer lymphocytes with desired specificities and effector functions, and to improve the *in vivo* efficacy and safety of adoptively transferred T cells.

This chapter focuses on the clinical promise of endogenous CTLs and TILs, as well as genetically modified TCR- and CAR-expressing T cells, for the treatment of cancers and viral infections. The limitations and safety concerns of each cell type will be debated, and an alternative process for producing antigen-specific CTLs, by differentiating stem cell into T cells, will be discussed. It was hypothesized that by using stem cells to generate functional T cells, the limitations associated with CTL and TIL isolation, and the dangers of using TCR- or CAR-modified T cells, could be minimized. The rational design of the proposed system will be presented in the biological context of T cell development and the various methods that are currently used to differentiate T cells *in vitro*.

2.1.1 General Approaches for the Isolation, Enrichment, and Expansion of Endogenous Adoptive Transfer T Cells

Significant progress has been made to optimize the isolation and expansion of antigen-specific CTLs or TILs, as well as to extend the *in vivo* persistence of *ex vivo*-manipulated T cells. In order to generate sufficient numbers of cells for infusion, T lymphocytes must first be isolated from a patient's resected tumor tissue or peripheral blood. In the case of resected tumors, TILs are disaggregated from the tissue and expanded *in vitro* for 4-8 weeks.[13,17] Patients are pre-conditioned with lymphodepleting agents such as cyclophosphamide or fludarabine, and after T cell transfer, high doses of IL-2 are given to increase clinical response rates (Figure 2.2).[13,17]

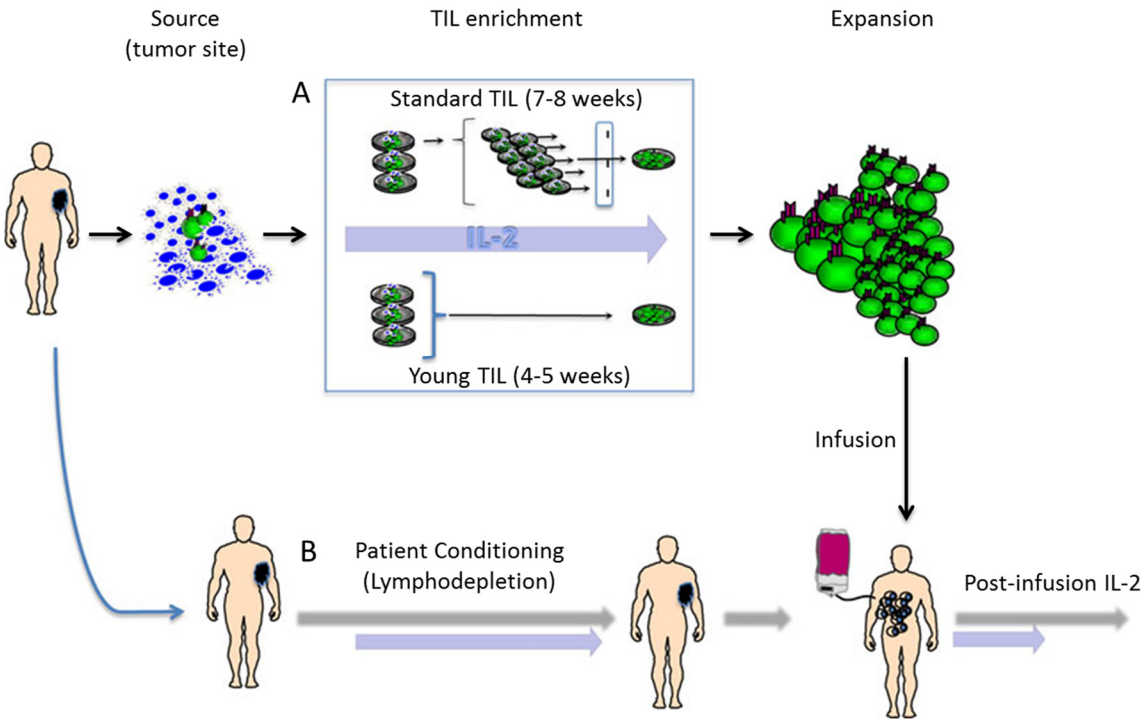

Figure 2.2: TIL therapy. TILs are dissociated from tumor tissue, followed by A) enrichment and expansion with IL-2 for 4-8 weeks. Young TIL cultures undergo shorter expansion periods, which are thought to yield cells with greater proliferative potential. B) Patients undergo lymphodepleting regimens prior to infusion. TILs are infused, and high doses of IL-2 are given post-infusion to enhance the tumor response.[†]

From the peripheral blood, antigen-specific T cells of interest are enriched and expanded using multiple rounds of stimulation with autologous epitope-presenting antigen-presenting cells (APCs).[13,17] Following enrichment, the cells are sorted by fluorescence activated cell sorting (FACS) or cloned by limiting dilution and further expanded using irradiated feeder cells in the presence of co-stimulatory factors, anti-CD3, anti-CD28, and interleukin (IL)-2 (Figure 2.3).[13,17] It takes approximately 6-14 weeks to generate sufficient numbers of cells required for each infusion (≥ 5 to 10

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billion).[13,17] Alternatively, the starting polyclonal T cell population can be non-specifically expanded using several rounds of stimulation with anti-CD3 and anti-CD28 antibodies.[13,17] The expanded population can be genetically modified with TCRs or CARs of interest, screened for effector function, and/or depleted of regulatory T (T_{REG}) cells to enhance the immune response.[13]

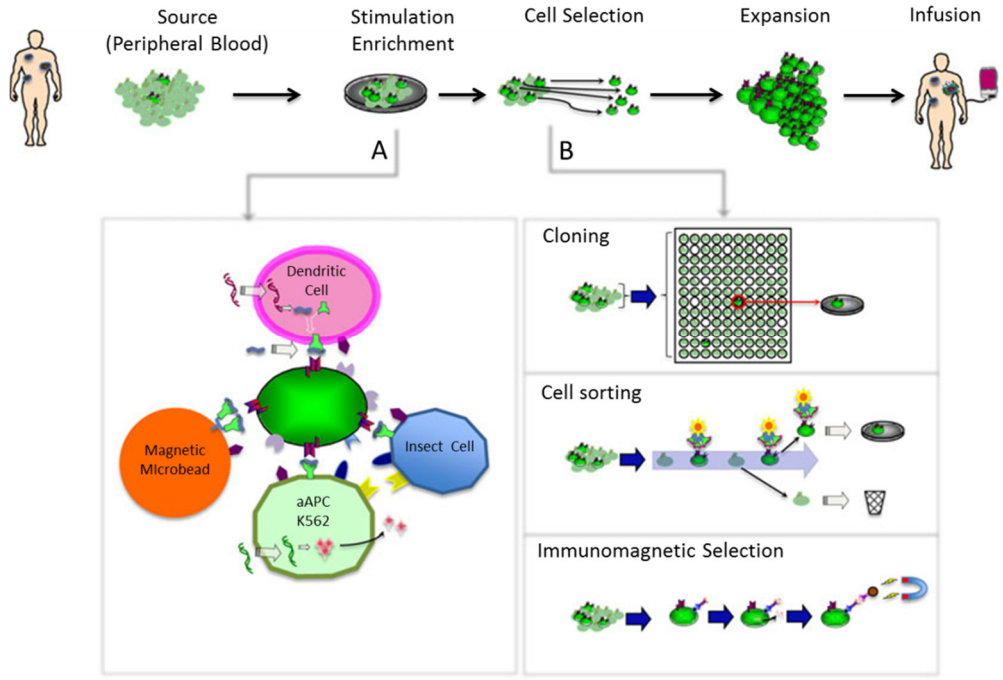

Figure 2.3: Circulating antigen-specific T cell therapy. A) CTLs are stimulated and enriched from isolated PBMCs using APCs and then B) selected for antigen-specificity by cloning or sorting. Cells are expanded by several rounds of stimulation before reinfusion.‡

The best results to date for the rapid expansion of CTLs utilizes irradiated allogeneic peripheral blood mononuclear cells (PBMCs).[24] While this method of expansion has been successful, it has been difficult to translate clinically. Due to patient

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variability and the inherent inefficiency of primary cell isolation, the scale-up of PBMCs for T cell expansion is expensive, tedious, and unable to conform to FDA-mandated requirements for validation. As a result, much effort has been given to the development of non-patient derived artificial APCs (aAPCs) capable of supporting large-scale adoptive transfer clinical trials.

Murine fibroblasts, engineered to express exogenous peptide human leukocyte antigen (pHLA) molecules and/or co-stimulatory molecules, have been used to expand melanoma antigen recognized by T cells (MART)-1-specific and glycoprotein (gp)100-specific CTLs.[25,26] Fc-expressing cell lines coated with co-stimulatory antibodies have also shown promise in stimulating polyclonal populations of T cells.[27] Acellular expansion methods, which can more easily conform to Good Manufacturing Practice, have also been fabricated and evaluated. The non-specific expansion of T cells has been shown using beads coated with anti-CD3 and/or anti-CD28 antibodies.[28] Additionally, microplates and microbeads, loaded with oligomeric forms of pHLA molecules, have shown promise in expanding various types of cancer and virus-specific CTLs, such as MART-1, gp100, and cytomegalovirus (CMV)-specific $CD8^+$ T cells.[29-31] These expanded cells have been shown to retain functionality and are capable of killing antigen-loaded target cells after *ex vivo* manipulation.

2.1.2 Antigen-Specific CTLs and TILs for Adoptive Transfer

Over the last two decades, the use of endogenous T cells, isolated from peripheral blood or tumor resections, as therapeutic agents in adoptive transfer immunotherapy has become increasingly promising. Numerous strategies to optimize isolation and expansion, direct functionality, and increase the *in vivo* efficacy and persistence of endogenous, antigen specific CTLs or TILs has made patient-specific adoptive transfer a clinical

reality. Adoptive T cell transfer has shown success in the treatment of renal cancer, leukemia, multiple myeloma, and prostate cancer, as well as PTLDs, such as CMV and Epstein-Bar Virus (EBV); however, some of the most well-studied and promising results have been seen in the treatment of melanoma.

2.1.2.1 Circulating Antigen-Specific T Cells and TILs for Treatment of Melanoma

The basis for adoptive T cell therapy as a treatment modality for cancer relies on the idea that a circulating population of tumor antigen-specific T cells exists *in vivo*. The earliest evidence for this was shown in metastatic melanoma patients who, when given high doses of immunomodulatory cytokines, such as interferon gamma (IFNγ) or IL-2, were more likely to exhibit clinical responses if they developed signs of autoimmune vitiligo.[32] The advent of peptide major histocompatibility complex (pMHC in mice, pHLA in humans) multimer technology facilitated the detection of antigen-specific T cell populations, and confirmed that populations of potentially reactive tumor antigen-specific T cells exist in melanoma patients.[33]

The first phase II clinical adoptive transfer trial for patients with melanoma was performed using a preparative dose of cyclophosphamide followed by high numbers of TILs (up to 2×10^{11}) and high-dose IL-2 infusions every 8 hours.[34] Eleven of the 20 patients in this study experienced tumor regression at multiple metastatic sites. Studies performed by Yee and colleagues using melanoma-specific CTL clones, isolated from PBMCs, have also shown promise in treating metastatic melanoma.[35] In their work, Chapius et al. expanded autologous MART-1 and gp100-specific T cell clones up to 20-fold to yield 10^{10} cells/m^2 for infusion.[35] Four infusions of CTLs with subsequent low-doses of IL-2 were administered to 10 patients, without serious toxicity.[35] 8 out of 10 patients maintained minor, mixed, or stable responses for up to 21 months, indicating that

adoptively transferred CTLs could mediate the elimination of antigen-expressing tumor cells, regression of metastases, and could persist *in vivo* when given with low doses of IL-2.[35]

2.1.2.2 Circulating Antigen-Specific T Cells for Treatment of Human CMV and EBV

Human CMV is a herpesvirus that infects 50-70% of the population. It is normally maintained in a latent state in immunocompetent hosts; however, in settings of T cell immunodeficiency, where immune responses to antigen exposure are impaired, primary infection or reactivation of the virus can progress to life-threatening conditions.[6,36] Patients with acquired immunodeficiency syndrome (AIDS), or those undergoing immunosuppressive regimens during allogeneic bone marrow or organ transplantation, are at increased risk.[6,36]

The use of CMV-specific T cells as safe, therapeutic agents in adoptive T cell transfer was demonstrated in a Phase I clinical trial performed by Riddell and colleagues. In this study, allogeneic bone marrow transplant recipients were given escalating doses of $CD8^+$ CMV-specific CTL clones isolated from three bone marrow donors.[37] These clones were screened for CMV-specificity, expanded *in vitro*, and infused into 14 patients weekly, for 4 weeks.[37] After 4 weeks of administration, 11 of the 14 patients exhibited CTL responses equivalent to that of donor peripheral blood lymphocytes (PBLs).[37] Analysis of TCR gene rearrangements confirmed that the donor-derived CTL clones were responsible for reconstitution and persisted *in vivo* for up to 12 weeks after infusion.[37] Interestingly, the CTL response declined in patients who did not reconstitute CMV-specific $CD4^+$ cells, suggesting that T helper (T_H) responses may be necessary for the long-term persistence of adoptively transferred CTLs. CMV disease was not detected in any of the 14 patients for the duration of the clinical trial.[37]

Significant therapeutic potential of adoptive T cell transfer has been demonstrated in EBV patients. Similar to CMV, EBV is a herpesvirus that is present in approximately 90% of adults. CTLs are responsible for controlling EBV, and induced immunosuppression can result in PTLD.[38] In a Phase 2 multicenter clinical trial, 33 patients bearing EBV epitope-expressing tumors were administered EBV-specific CTL clones weekly for 4 weeks.[38] Consistent with the hypothesis that $CD4^+$ T_H cells may sustain CTL activity, better responses were seen in patients receiving CTLs containing higher percentages of $CD4^+$ cells. Seventeen of the 33 recipients showed a tumor response rate 6 months after administration.[38]

2.1.2.3 Limitations of Patient-Isolated Antigen-Specific T Cells or TILs

Despite the success of adoptively transferred TILs and antigen-specific T cells to mitigate viral disease and induce tumor regression, limitations associated with the use of these cells have prevented adoptive transfer from becoming a more widely used clinical therapy. It is often difficult to isolate enough tumor-specific T cells to mediate a therapeutic response. In addition, T cells isolated from patients bearing tumors or chronic viral infections are often sub-functional due to immunosuppression by the tumor microenvironment or from chronic antigen stimulation. Increasing evidence has demonstrated that T cells can become exhausted or senescent as a result of immunosuppressive signals presented by tumor cells, or by constant antigenic stimulation. Thus, the efficiency of isolating functional TILs or antigen-specific T cells varies from patient-to-patient and is only successful for some people. Even if sufficient numbers of T cells can be isolated, it is often difficult and time-intensive to expand CTL clones to sufficient numbers for adoptive transfer. For patients with advanced stages of

disease, this is not optimal and has contributed to the high attrition rates of adoptive T cell transfer clinical trials.

2.1.3 TCR Gene-Modified T Cells, CARS, and TRUCKs

Due to the inherent complications of using endogenous CTLs or TILs, many researchers have shifted their efforts to developing T cells with pre-defined antigen-specificities *ex vivo*, either using high-affinity TCRs or CARs (Figure 2.4).[11,18]

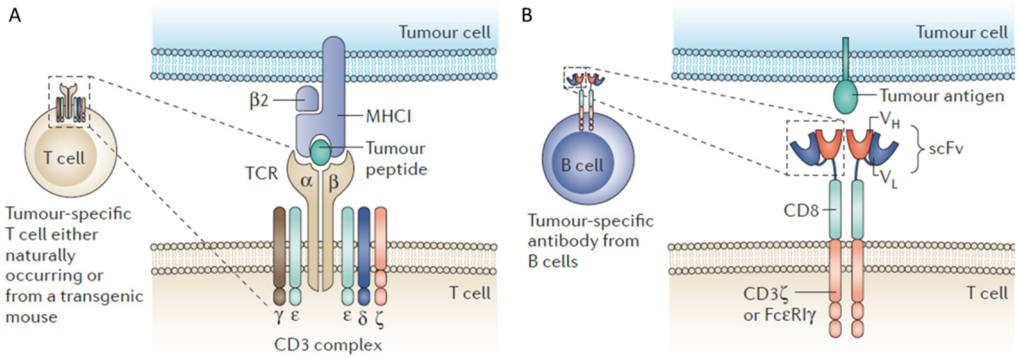

Figure 2.4: Gene-modified T cell therapy targeted against tumor antigens. A) High-affinity αβTCRs can be isolated from tumor-specific T cells or from HLA-transgenic mice. B) CARs consist of an antibody-derived scFv, an extracellular linker, a transmembrane domain, and an intracellular T cell-derived signaling domain.[§]

2.1.3.1 TCR Gene-Modified T Cells

The process of TCR gene transfer has advantages over traditional T cell adoptive transfer. First, it eliminates the expensive and time-consuming process of screening for patient-derived antigen-specific T lymphocytes.[11,18,39,40] Additionally, TCR affinity, avidity, and stability can be finely optimized to engineer cells with unique functional capabilities.[11,18,39,40] Finally, TCR gene-modified T cells facilitate the production of clinically feasible numbers of antigen-specific T cells in a short amount of time.[11,18,39,40]

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The majority of TCR-engineered cells that have been clinically evaluated are directed against melanoma antigens (MART-1, gp100, carcinoembryonic antigen (CEA), and melanoma-associated antigen (MAGE)-A3).[41] Results from these trials indicate that TCR-engineered cells can successfully induce tumor response in patients with metastatic melanoma, and can even successfully treat melanoma brain metastasis. However, these clinical trials also highlight potential toxicity of engineered TCRs, especially if they target antigens present on tumors and normal tissue.[41] In studies testing MAGE-A3 engineered TCRs, two patients fell into comas and died, most likely due to the recognition of neurons expressing self-antigens, MAGE-A12 and MAGE-A9, by the TCR-directed MAGE-A3-specific CTLs.[42]

Such findings indicate the need for further preclinical optimization and characterization of TCRs to reduce cross-reactivity and increase specificity of targeting. Vectors have been constructed with various promoters, internal ribosome entry site (IRES) elements, and peptide linkers to maximize expression and optimize proper TCRα- and TCRβ- pairing in transduced T lymphocytes.[43] High-avidity T cells have been generated by enhancing TCR transgene expression levels using TCRα- and TCRβ- chain genes which have had mRNA instability motifs and cryptic splice sites removed.[44] Additionally, high-avidity TCRs can be isolated from allo-specific human T cells or from HLA-transgenic mice (Figure 2.4A).[44]

2.1.3.2 CARs and TRUCKs

CAR-modified T cells are attractive alternatives to endogenous T cells or TCR-engineered cells in adoptive transfer therapies.[18,39] Unlike TCRs, CARs consist of a polypeptide chain with a tumor-associated antigen (TAA)-binding domain, derived from antibody single-chain variable fragments (scFvs), an extracellular hinge region, a

transmembrane domain, and an intracellular signaling chain, normally the TCR-derived CD3ζ chain (Figure 2.4B).[18,39] As a result, CARs can recognize antigens in a pMHC/HLA-independent manner, allowing them to be used universally in patients, regardless of HLA-type.[18,39]

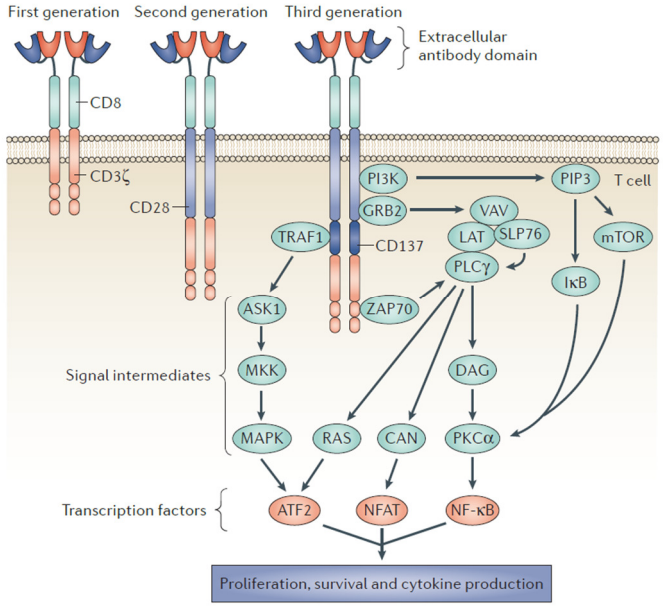

Figure 2.5: First, second, and third generation CARs. First generation CARs contain a single intracellular signaling domain of T cell-derived CD3ζ subunits. Second generation CARs contain the intracellular portion of co-stimulatory molecule, CD28, and third generation CARs contain an additional signaling unit.** See Section 2.7 for list of abbreviations.

Upon antigen engagement by the CAR, activation of CD3ζ-associated kinases results in T cell stimulation, cytokine production, and increased cytotoxicity (Figure 2.5).[39] Second generation CARs have been modified to express co-stimulatory domains of CD28 in addition to CD3ζ in order to prolong T cell activation (Figure 2.5).[39] Third generation CAR T cells have been engineered to contain additional co-stimulatory B7

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(i.e. inducible T cell co-stimulator (ICOS)) or tumor necrosis factor receptor (TNFR) family members (i.e CD137, CD134), and have shown enhanced anti-tumor activity compared to first generation CARs (Figure 2.5).[39]

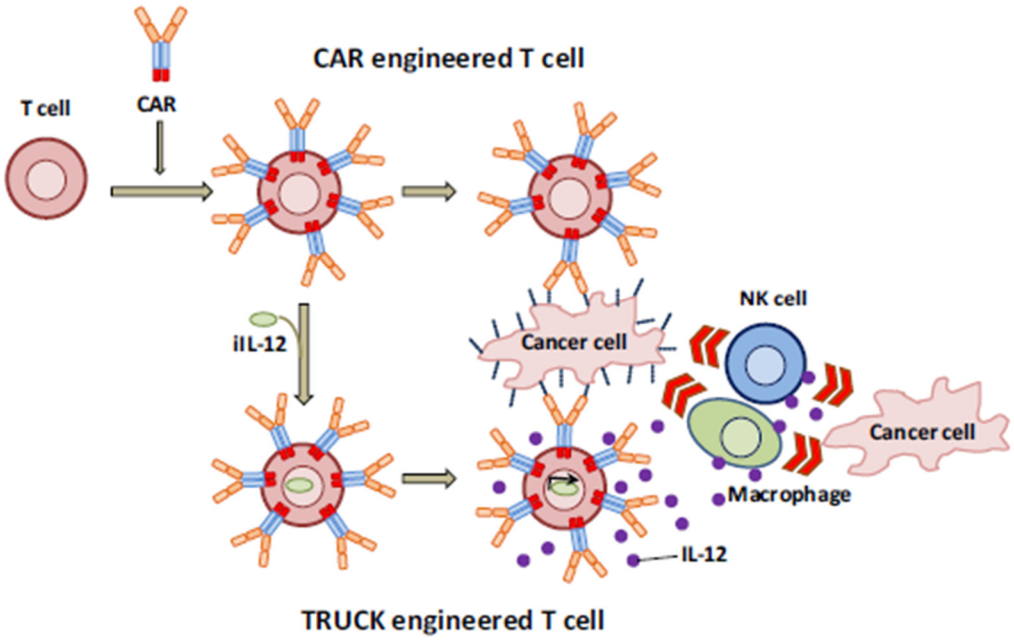

Figure 2.6: Differences between CARs and TRUCKs. TRUCKs are CARs that have been redirected to secrete cytokines upon antigen-activation. Inducible IL-12 (iIL-12)-secreting TRUCKs produce IL-12 locally, which attracts cells of the innate immune system to enhance the anti-tumor response.††

To combat the effects of antigen loss on tumor cells, as well as the immunosuppressive effects of T_{REG} cells or myeloid derived suppressor cells (MDSCs), CAR-derived T cells known as TRUCKs (T cells redirected for universal cytokine-mediated killing) have been generated (Figure 2.6).[18] TRUCKs are CAR T cells that have been engineered to release transgenic pro-inflammatory cytokine products at the site of

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targeted tissue. This local accumulation of cytokines attracts a second wave of immune cells that can attack cells which are not targeted by the CAR T cells.[18] The feasibility of this technology has been demonstrated using IL-12-expressing TRUCKs. Studies by Chmielewski and colleagues have shown that IL-12-secreting TRUCKs can recruit natural killer (NK) cells and macrophages to tumor tissue, resulting in enhanced local killing.[18] Additional studies have shown that IL-12-secreting TRUCKs have the capacity to revert immune suppression induced by MDSCs or chronic infection, to an inflammatory environment.[18]

2.1.3.3 Limitations and Safety Concerns of TCR-Gene Modified T Cells, CARs, and TRUCKs

Re-directing T cell specificity using high-affinity or high-avidity TCRs is a promising strategy for adoptive transfer immunotherapy because it eliminates the need to screen for patient antigen-specific T cells and allows one to fine tune T cell responses. However, re-directed T cells pose several risks. Recent studies indicate that hybrid TCRs between exogenous and endogenous TCR chains can form. This could alter the desired specificity, resulting in off-target effects and potentially auto-reactive T cells.[18,39,43] Transduction of hematopoietic stem cells (HSCs) with TCR genes controlled by endogenously active promoters facilitates early, exclusive expression of the exogenous TCR and helps to circumvent the risk of autoimmunity.[45] However, genetic modification of HSCs or T cells with retroviral vectors still poses the threat of insertional mutagenesis, potentially resulting in lymphoma.[18,39,43]

Similar concerns apply to CAR-modified T cells. Unexpected targeting may occur *in vivo*, and insertional mutagenesis during genetic modification poses safety concerns. In addition, systemic inflammatory reactions known as cytokine release syndrome (CRS), have been reported.[13,18,39-41] CRS is caused by an acute systemic immune response

mediated by T cells, B cells, NK cells, and macrophages which release large amounts of cytokines.[13,18,39,41] This response is seen in GVHD, monoclonal antibody treatment, and during severe bacterial and viral infections, and can lead to organ damage, heart failure, and in some cases death. CRS has been observed in clinical trials utilizing CD19- or CD20-specific CAR T cells to treat hematologic malignancies.[11,19-21] Out of six clinical trials reporting CRS, two-thirds of patients who were administered CAR T cells experienced CRS within 6-20 days post-infusion.[46,47] Two cases have reported fatalities. Corticosteroids and immunosuppressive cytokines are currently used to alleviate symptoms of CRS.[18,19,46,47] Efforts to minimize CRS occurrence during CAR T cell therapy have been proposed, including the in-depth quantification of cytokine levels and identification of cytokine polymorphisms that may make patients more susceptible to CRS.[18,19,46,47] Additionally, a conservative dose-escalation strategy has been implemented for many Phase I clinical trials, and the design of short-lived or less-differentiated CAR T cells is being pursued.[18,19,46,47]

2.1.4 Optimal Extrinsic and Intrinsic Conditions for *Ex Vivo* Expanded T Cells

Extrinsic conditions, such as preemptive lymphodepletion or nonmyeloablative conditioning, have been shown to enhance the efficiency of adoptively transferred T cells, either by eliminating suppressor T_{REG} cells and/or MDSCs, or by reducing competition for homeostatic cytokines, IL-7 and IL-15.[48,49] In studies done by Gattinoni et al., the genetic deletion of T_{REG} cells significantly enhanced anti-tumor efficacy of adoptively transferred CTLs.[48] By systematically deleting γc cytokine-responsive cells, Gattinoni and colleagues showed enhanced anti-tumor responses due to increased CTL functionality, but not number.[48] However, genetic deletion of IL-7 and IL-15 abrogated CTL effectiveness.[48] Their data indicates that lymphodepletion enhances the functionality

of adoptively transferred CTLs by removing endogenous cells that compete for cytokines. These findings have been corroborated by Wrzesinksi et al., who have demonstrated that the intensity of lymphodepletion and the anti-tumor efficacy of adoptively transferred CTLs are positively correlated.[49]

The subsets of $CD8^+$ T cells that are available for adoptive transfer express distinct surface markers and differ greatly in terms of function and homing ability. One crucial limitation of adoptive T cell transfer has been the inability of T lymphocytes to persist and maintain functionality *in vivo*. Recent studies to identify T cell subsets, including naïve (T_N) T cells, antigen-experienced central memory (T_{CM}) and effector memory (T_{EM}) T cells, which are capable of *in vivo* persistence, indicates that the selection of T cells prior to adoptive transfer is critical.[50] Riddell and colleagues have demonstrated that effector cells derived from $CD62L^{hi}$ T_{CM} cells have the capability of re-expressing the molecules required for homing and persisting in the lymphoid organs. $CD62L^{hi}$ T_{CM} cells are also capable of establishing reservoirs of T_{CM} and T_{EM} cells, which are necessary for long-lasting immune reconstitution.[50] These findings demonstrate that the intrinsic properties of T cells are important factors to consider when screening T lymphocytes for adoptive transfer.

2.2 DIRECTION OF THIS TREATISE

Numerous clinical studies have shown the potential of adoptively transferred T cells to treat malignancies and viral diseases, and much research has gone towards developing new targeting strategies, as well as to understanding the biology behind T cell transfer. These findings have undoubtedly enhanced the field of immunotherapy; however, they also highlight the limitations and safety concerns associated with using endogenous or genetically modified T cells. As a result, a high-throughput method for

generating antigen-specific CTLs, without the unforeseen risks of genetic modification, is needed. It was hypothesized that stem cells could serve as renewable sources for adoptive transfer T cells. To support this hypothesis, the biology behind T cell development and the successes of many groups to generate T cells from stem or progenitor cells *in vitro* are discussed in Sections 2.3 and 2.4.

2.3 T CELL DEVELOPMENT IN THE THYMUS

2.3.1 Stages of T Cell Development

T cells are unique lymphocytes that develop primarily in the thymus. The unique microenvironment within the thymus provides signals that drive the generation of a diverse repertoire of self-restricted and self-tolerant T cells. Thymic seeding progenitors (TSPs) enter the thymus from the adult bone marrow at the cortical-medullary junction. There, the progenitors undergo T lineage commitment followed by a series of well-characterized phenotypic changes (Figure 2.7).[51,52] Initially, the cells are double negative (DN) for CD4 and CD8 ($CD4^-CD8^-$), and are subdivided based on the presence of CD25 and CD44. DN1 cells ($CD44^+CD25^-$) have the potential to become αβ T cells, γδ T cells, dendritic cells (DCs), macrophages, or B cells.[53]

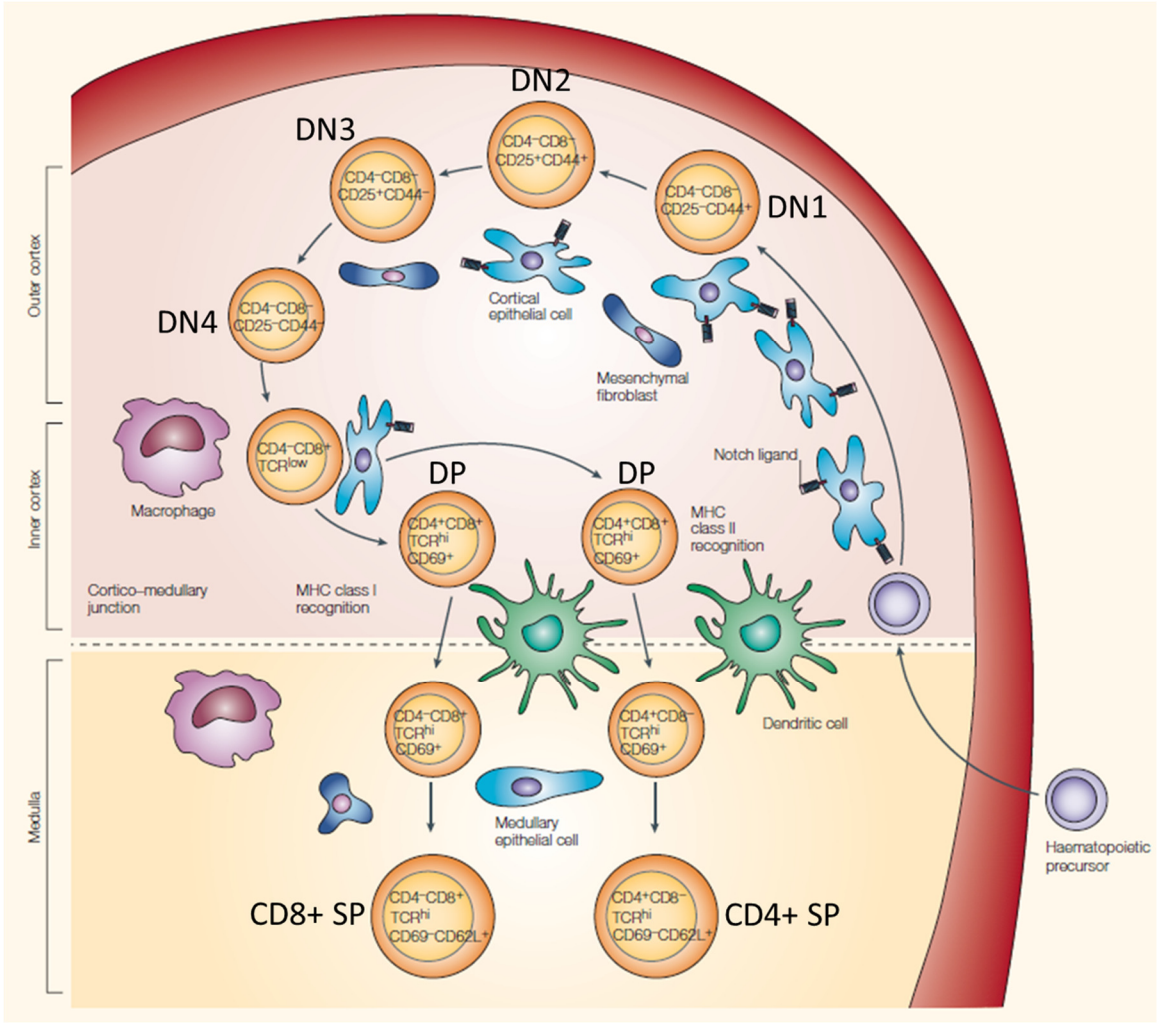

Figure 2.7: T cell development within the thymus. Hematopoietic progenitors migrate from the bone marrow to the thymus. Notch signaling induces T lineage commitment and differentiation through the DN1-DP stage. pMHCs on the surface of APCs induce positive and negative selection, resulting in a repertoire of self-restricted, self-tolerant $CD4^+$ or $CD8^+$ T cells.‡‡

After T lineage specification, DN1 cells become DN2 cells ($CD44^+CD25^+$) and undergo TCR-β, TCR-γ, and TCR-δ rearrangements.[51,52] The transition from DN2 to DN3 ($CD44^-CD25^+$) indicates full commitment to the T cell lineage.[51,52] DN3 cells

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express pre-TCRs consisting of a successfully rearranged TCRβ chain, associated with invariant TCRα and invariant CD3 signaling molecules. The expression of a productive pre-TCR rescues these precursors from apoptosis and initiates proliferation.[51,52] The thymocytes then transition to the DN4 ($CD44^-CD25^-$) stage, followed by an up-regulation of CD4 and CD8, which characterizes double positive (DP) $CD4^+CD8^+$ thymocytes.[51,52] The TCRα-chain of DP cells rearranges and pairs with the β-chain to produce a TCRαβ heterodimer.[51,52]

Approximately 98% DP thymocytes will undergo apoptosis during a series of developmental checkpoints known as positive and negative selection. During positive selection, DP thymocytes, which primarily inhabit the thymic cortex, undergo "death by neglect" if their αβTCRs fail to engage self pMHCs, presented by cortical thymic epithelial cells (cTECs), with appreciable affinity.[51,52] Cells that bind with intermediate affinities to self pMHC Class I or Class II molecules mature into single positive (SP) $CD8^+$ or $CD4^+$ cells, respectively. SP T cells migrate to the medulla, where they come into contact with $Aire^+$ medullary TECs (mTECs) expressing tissue-restricted antigens (TRAs).[51,52] Thymocytes that bind with high affinity to these self-antigens undergo negative selection and are eliminated from the repertoire. Thymocytes that pass both checkpoints exit the thymus as self-restricted and self-tolerant mature $CD8^+$ or $CD4^+$ T cells.[51,52]

2.3.2 Notch-DLL and TCR-pMHC Signaling in T Lymphocyte Development

The development of T cells within the thymus is a highly controlled and well-orchestrated process dependent on two sequential signals: 1.) Notch receptor-Delta like ligand (DLL) signaling and 2.) TCR-pMHC signaling.[51,52] The quality and quantity of

these two cues are critical for T lymphoid commitment, progression through the various developmental stages, and the production of self-recognizing, non-autoimmune T cells.

2.3.2.1 Notch Signaling in T Lymphopoiesis

Notch receptors are highly conserved heterodimeric transmembrane proteins that are essential for transducing signals that control cell fate decisions, including T cell development. Mammalian Notch receptors (Notch 1-4) recognize the DSL ligands (Delta, Serrate/Jagged, Lag), and upon ligand binding, the extracellular portion of the Notch receptor undergoes proteolytic cleavage by a disintegrin and metalloproteinase (ADAM).[51,54-59] The second proteolytic cleavage of Notch, mediated by γ-secretase, cleaves the intracellular portion, which translocates to the nucleus and interacts with C-promoter binding factor 1 (CBF1) to promote the transcription of hairy and enhancer of split (HES) gene products (Figure 2.8).[59]

Notch signaling is essential for proper T lymphopoiesis. Studies have shown that deletion of Notch-1 in bone marrow stem cells results in a population of immature thymocytes that express markers similar to B cell precursors of the bone marrow; however, the absence of Notch-1 does not affect the development of B cells in the bone marrow or spleen.[56,60-63] Conversely, gain-of-function experiments show that bone marrow stem cells with constitutively expressed Notch-1 give rise to T lineage-committed cells expressing CD4, CD8, Thy1, CD3 and TCRβ in the bone marrow.[56,60-63] Constitutively expressed Notch-1 also prevents bone marrow stem cells from differentiating into B cells, suggesting that Notch signaling inhibits B lineage commitment, which may be sufficient to induce T cell lineage commitment.[56,60-63]

In the context of the thymus, Notch-1 receptors on the surface of lymphoid progenitors interact with DLLs presented by TECs. While both DLL1 and DLL4 are

expressed by TECs, conditional deletion experiments have shown that only DLL4 is essential for T cell development.[64-66] When ectopically expressed on OP9 stromal cells, lower levels of DLL4 than DLL1 are needed to induce T cell development.[67] However, it has been shown that both DLL1- or DLL4-expressing stromal cells are capable of inducing Notch activation and T lymphopoiesis *in vitro*.[67]

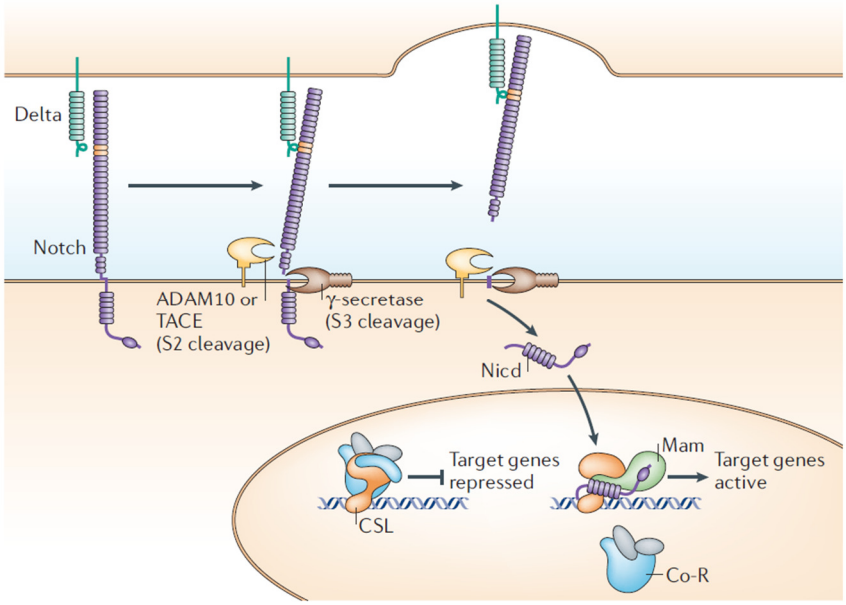

Figure 2.8: Notch-Delta (like) ligand binding induces cleavage of extracellular Notch via ADAM, followed by cleavage of intracellular Notch by γ-secretase. Intracellular Notch translocates to the nucleus and binds to the CSL (Cbf1, Su(H), and LAG-1) protein, facilitating transcription of HES genes.[§§]

2.3.2.2 TCR-pMHC Signaling in T Lymphopoiesis

TCR-pMHC signaling strength is known to drastically influence the fate and survival of developing thymocytes, both in the context of positive and negative selection, and the CD4 versus CD8 lineage decision.[68,69] TCR-pMHC interactions span a wide range of affinities due to the diversity of TCRs and self-peptides displayed on each MHC.

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It is well-established that the strength of TCR signaling determines qualitatively different cell fates during development. Experiments using TCR transgenic mice have shown that a narrow affinity window for positive versus negative selection exists and slight alterations in affinity can change fate of immature thymocytes.[70-74] Recent work done by Moran and colleagues has shown that T cells undergoing negative selection receive stronger TCR signals compared to cells undergoing positive selection, and $CD4^+FoxP3^+$ T_{REG} cells perceive stronger TCR signals than conventional $CD4^+$ T cells during development.[73,74]

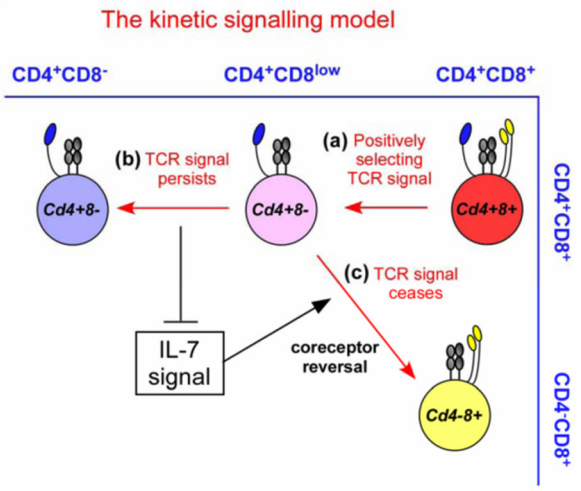

Figure 2.9: The kinetic signaling model used to explain CD4/CD8 lineage commitment. According to this theory, DP thymocytes that receive positive selecting/survival signals downregulate CD8 expression to yield ISP $CD4^+CD8^{low}$ cells. The absence of Class I signaling results in IL-7-induced coreceptor reversal and directs cells to the $CD8^+$ lineage. Sustained signaling from Class II molecules blocks IL-7R signaling, and results in $CD4^+$ commitment.***

Several models have been presented to explain how strength of signal influences the CD4/CD8 lineage commitment.[69] An early model, termed the stochastic selection

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model, suggested that the termination of co-receptor gene expression was a random event, and only thymocytes with matching TCRs and co-receptors survived.[75,76] According to the instructive strength- or duration-of-signal models, differences in signal strength or duration, transduced via the TCR and co-receptor, instruct lineage commitment, with stronger/longer signals directing $CD4^+$ lineage commitment and weaker/shorter signals directing $CD8^+$ lineage commitment.[77-82]

There has been much debate over the accuracy of these models. The most recent findings suggest that IL-7R signaling acts as a sensor for TCR signaling (Figure 2.9).[69,83] According to the kinetic signaling model, $CD4^+CD8^+$ DP cells that receive positively selecting signals downregulate CD8 expression to become intermediate single positive (ISP) $CD4^+CD8^{low}$ cells.[69] Loss of signal from Class I molecules at the ISP stage permits IL-7 signaling, which induces coreceptor reversal and directs cells to the $CD8^+$ SP lineage.[69] Conversely, sustained signaling from Class II molecules during the ISP $CD4^+$ $CD8^{low}$ stage blocks IL-7 signaling and results in $CD4^+$ SP commitment. This phenomenon, as well as the selection and instruction models, is discussed in more detail in Chapter 5.[69]

2.4 CURRENT T CELL DIFFERENTIATION SYSTEMS

2.4.1 *Ex Vivo* Methods to Study T Cell Development

2.4.1.1 Fetal Thymus Organ Culture and Reaggregate Thymus Organ Culture

Many systems to study T cell development and generate T cells *ex vivo* have been explored, the earliest of these being fetal thymus organ culture (FTOC). FTOC has been a seminal tool for studying T cell development and the role of thymic stromal cells in T lymphopoiesis.[84] This system involves the isolation and *in vitro* maintenance of embryonic thymic lobes.[84] Modifications of FTOC, such as hanging drop cultures or

reaggregate thymus organ culture (RTOC), in which defined cohorts of thymocytes and/or stromal cells are cultured together, has provided much insight into specific stages of T cell development.[84] However, from a clinical standpoint, using FTOC and RTOC to generate transplantable T cells is not feasible.

2.4.2 *In Vitro* Methods to Study T Cell Development

2.4.2.1 OP9 Cells for In Vitro Hematopoiesis and B Cell Development

Nakano and colleagues were the first group to show that the bone marrow stromal cell line, OP9, could direct undifferentiated embryonic stem cells (ESCs) into the erythroid, myeloid and B cell lineages.[85] Later efforts by Cho et al. showed that the addition of FMS-like tyrosine kinase 3 ligand (Flt3L) to the OP9/ESC co-culture enhanced B lymphopoiesis and the proliferation of B cell progenitors.[86] The temporal progress and phenotypic characteristics of ESC-derived B cell precursors in the OP9/ESC system were analogous to that of progenitor and mature B cells *in vivo*.[86] Although OP9 co-cultures were successful in producing multiple types of hematopoietic cells *in vitro*, T cells could not be generated because OP9 cells do not express the Notch ligands necessary for T cell commitment and differentiation.

2.4.2.2 Notch Ligand-Expressing OP9 Cells for Murine T Cell Development

Efforts to generate T cells *in vitro* using Notch ligand-transfected OP9 cells were pioneered by Dr. Zúñiga-Pflücker and colleagues. Schmitt et al. developed OP9 cells expressing high levels of DLL1 via retroviral transfection (OP9-DL1) (Figure 2.10).[57] Initial experiments showed that co-culture of murine fetal liver $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ (LSK) hematopoietic progenitor cells (HPCs) with OP9-DL1 cells resulted in the development of $CD4^{+}CD8^{+}$ immature DP T cells and a small population of $CD8^{+}$ SP T cells after 12 days, similar to the time frame following transfer of HPCs into FTOC.[57] In HPC-derived

co-cultured T cells, rearrangement of the TCR-β locus was similar to that occurring in fetal thymocytes.[57] $CD8^+$ SP T cells isolated from co-cultures could respond to plate-bound anti-CD3 and anti-CD28, and were capable of activation-induced proliferation and IFNγ production.[57]

Schmitt et al. have also demonstrated that murine ESCs are able to differentiate into functionally mature $CD8^+$ T cells using OP9-DL1 co-culture (OP9-DL1/ESC). Importantly, ESC-derived T cell progenitors can reconstitute immunodeficient mice and mount a functional immune response.[87] In their studies, $CD25^+CD44^+$ and $CD25^+CD44^-$ DN cells were sorted from OP9-DL1/ESC co-cultures and transferred into fetal thymic lobes that were then transplanted under the skin of recombination activating gene (Rag)$2^{-/-}$ mice.[87] Analysis of the spleens and lymph nodes showed reconstitution of TCR^+CD4^+ and TCR^+CD8^+ T cells. $Rag2^{-/-}$ mice reconstituted with ESC-derived T cell progenitors were also able to mount an immune response similar to that of normal C57BL/6 mice after infection with lymphocytic choriomeningitis virus (LCMV).[87]

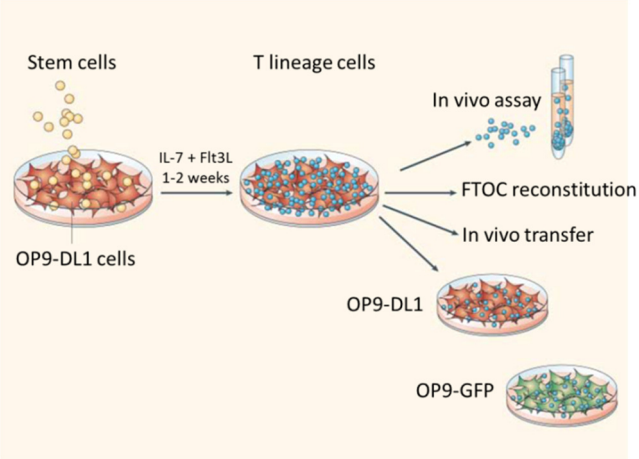

Figure 2.10: Schematic of OP9-DL1/stem cell co-culture.†††

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2.4.2.3 Notch Ligand-Expressing OP9 Cells for Human T Cell Development

La Motte-Mohs et al. have used the OP9-DL1 co-culture system to differentiate human cord blood HSCs into T cells. $CD34^+CD38^-$ HSCs cultured on OP9-DL1 monolayers rapidly express CD38 and follow the normal steps of human T cell differentiation including the generation of $CD7^+CD1a^+$ pre-T cells, $CD4^+CD8^+$ DP cells, and $CD4^+$ and $CD8^+$ SP T cells.[55] Further studies indicate that human $CD8^+$ SP T cells generated from OP9-DL1 co-cultures are functionally equivalent to *ex vivo* thymus-derived T cells and bear a diverse TCRVβ repertoire.[55] Pro-T progenitors from OP9-DL1 co-cultures showed successful engraftment when transferred into immunocompromised NOD/SCID/γc^{null} or BALB/c $Rag2^{-/-}\gamma c^{-/-}$ mice, as CD3-expressing $CD4^+CD8^+$ DP cells, $CD4^+$ SP, and $CD8^+$ SP cells were found 4-6 weeks after transplantation.[88]

2.4.2.4 Antigen-Specific and Self-Tolerant T Cells from OP9-DL1 Co-Cultures

Results show that an antigen-specific response can be initiated by *in vitro*-derived murine $CD8^+$ T cells from OP9-DL1 co-cultures. Bulk $CD8^+$ cells from OP9-DL1 co-cultures were stimulated with DCs presenting model antigens, gp33 or human tyrosinase-related protein (hTrp)2.[89] For both epitopes, the frequency of antigen-specific T cells were similar to those observed in *ex vivo* $CD8^+$ T cells at the time of peak response, 14 days after DC stimulation, demonstrating the ability of *in vitro*-derived cells to mount a normal antigen response.[89] Additionally, mixed lymphocyte reaction (MLR) assays indicate that *in vitro*-derived T cells are self-tolerant. When $CD8^+$ T cells from OP9-DL1 cultures were stimulated with irradiated, syngeneic PBMCs, no proliferation was detected.[89] As expected, $CD8^+$ T cells cultured with allogeneic splenocytes displayed increased proliferation. Upon transfer of *in vitro*-derived, carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD8^+$ T cells into syngeneic mice, no proliferation was observed, confirming a loss of self-reactivity.[89] However, loss of CFSE was

observed when $CD8^+$ T cells were transferred into allogeneic mice, suggesting that *in vitro*-derived T cells underwent positive and negative selection to yield a functional, yet self-tolerant repertoire.[89]

2.4.2.5 Limitations of OP9-DL1 Co-Cultures

While promising, the therapeutic use of the OP9-DL1 co-culture systems is complicated by several factors. The inherent nature of co-culture systems poses difficulties for large scale T cell production, especially in 3D biomimetic scaffolds or bioreactor cultures. Using genetically-modified feeder cells complicates clinical applications because they must be separated to yield a pure T cell population, free of contaminating stromal cells. Recent studies have also demonstrated the dependence of Notch ligand dose on T cell differentiation. By using a cell-based system, such as OP9-DL1 co-culture, it is difficult to control and validate the amount of Notch ligand presented to progenitors in mixed cultures.

2.4.3 Immobilized Notch Ligands for T Cell Differentiation

2.4.3.1 Plate-Immobilized Notch Ligands Induce Early Murine T Cell Development in a Dose-Dependent Manner

Several reports indicate that plastic plates, immobilized with DLL1, along with a defined combination of cytokines, can induce Notch signaling and early T cell development of mouse LSK cells and human $CD34^+CD38^-$ HPCs in a dose-dependent manner. Dallas et al. showed that culture of mouse LSK cells with high densities ($\geq$ 1.25 μg/mL) of immobilized DLL1 resulted in a 4-log increase of $Thy1^+CD25^+$ T cell precursors compared to control cultures.[90] After 28 days of culture with Notch ligand-coated plates, CD3 and TCRα expression was significantly higher compared to control cultures, and increased in a dose-dependent manner.[90] High densities of immobilized

Notch ligand inhibited B lineage commitment, as the number of $B220^{+}CD43^{-/low}$ B cell precursors increased when lower densities of DLL1 was used ($\leq$ 1.25 μg/mL).[90]

2.4.3.2 Lymphoid Commitment of Human Cord Blood $CD34^{+}$ Cells Using Plate-Bound Notch Ligands

Delaney et al. demonstrated that lymphoid commitment of human $CD34^{+}CD38^{-}$ HPCs is dependent on Notch ligand DLL1 density. Culture of $CD34^{+}CD38^{-}$ progenitors with low densities of immobilized DLL1 (< 5 μg/mL) resulted in a 5-fold increase in $CD7^{+}$ lymphoid-committed cells compared to the control cultures after 21 days.[91] A progressive increase in $CD7^{+}$ lymphoid cells was observed with increasing concentrations of DLL1, suggesting that lymphoid maturation is density-dependent.[91] After 10 days of culture with high concentrations of Notch ligand (> 5 μg/mL), a DN2 population ($CD44^{+}CD25^{+}$) was observed.[91] This population was shown to increase significantly as a function of Notch ligand density. After 14 days of culture with high concentrations of Notch ligand, a 5-10 fold increase of cytoplasmic CD3ε was observed in progenitor cells.[91]

2.4.3.3 Bead-Immobilized Notch Ligands can Induce T Cell Development without Stromal Cell Contact

Recently, Taqvi et al. showed that insert co-cultures employing Notch ligand-functionalized microbeads can induce the differentiation of mouse bone marrow LSK cells into early $Thy1.2^{+}$ T cells.[92] Cells differentiated with control microbeads differentiated into B lineage-committed $CD19^{+}$ cells (Figure 2.11).[92] This supports the idea that artificially-presented Notch ligands are sufficient to support T cell development and that stromal cell contact is not necessary, as long as the appropriate soluble factors are provided during differentiation.[92] Based on these data, it was hypothesized that early human T cells could be generated by culturing $CD34^{+}CD38^{-/low}$ progenitor cells with

immobilized DLL1 in stromal cell-conditioned media. In this dissertation, the generation of human $CD1a^+CD7^+$ and $CD4^+CD8^+$ early T cells using immobilized Notch ligands and stromal cell-secreted soluble factors is demonstrated.

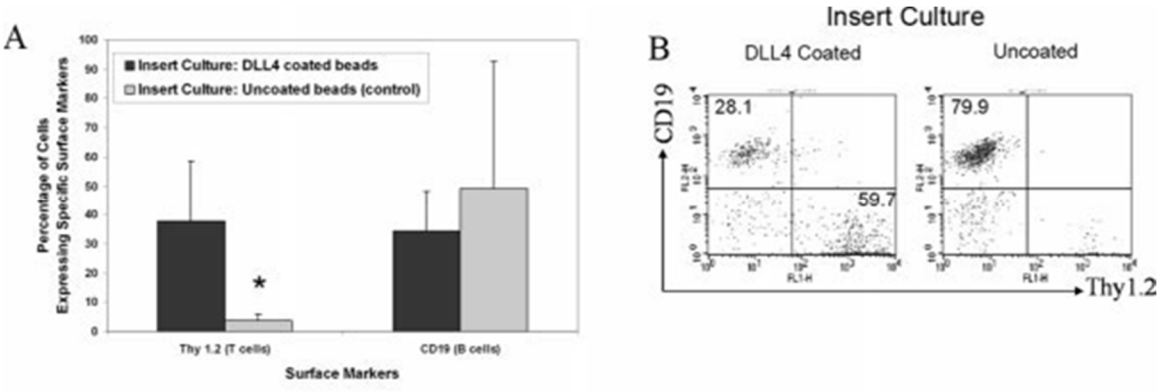

Figure 2.11: Notch ligand-coated microbeads induce T lineage commitment in the presence of secreted stromal factors. A,B) DLL4-coated beads induced significantly higher percentages of $Thy1.2^+$ cells from bone marrow LSK cells compared to control beads, in cultures free of stromal cell contact.‡‡‡

2.4.3.4 Generation of Antigen-Specific $CD8^+$ SP T Cells from Murine Progenitors Using pMHC Class I Tetramers

Lin et al. previously demonstrated that pMHC Class I tetramers can induce the development of antigen-specific T cells from murine DP thymocytes and ESC-derived early T cells. $CD4^+CD8^+$ DP thymocytes and early T cells, generated from OP9-DL1/ESC co-cultures, were cultured with LCMV.GP34p-MHC Class I tetramers in the presence of co-stimulatory molecules tetramers for 7 days.[93] Both groups of progenitor cells differentiated into $CD8^+$ LCMV.GP34-specific T cells after the 7-day time period (Figure 2.12).[93] Importantly, these cells exhibited specific killing ability against epitope-presenting target cells *in vitro*. In this dissertation, the differentiation of human HSC-

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derived early T cells into functional, antigen-specific $CD8^+$ T cells using pHLA Class I tetramers is demonstrated.

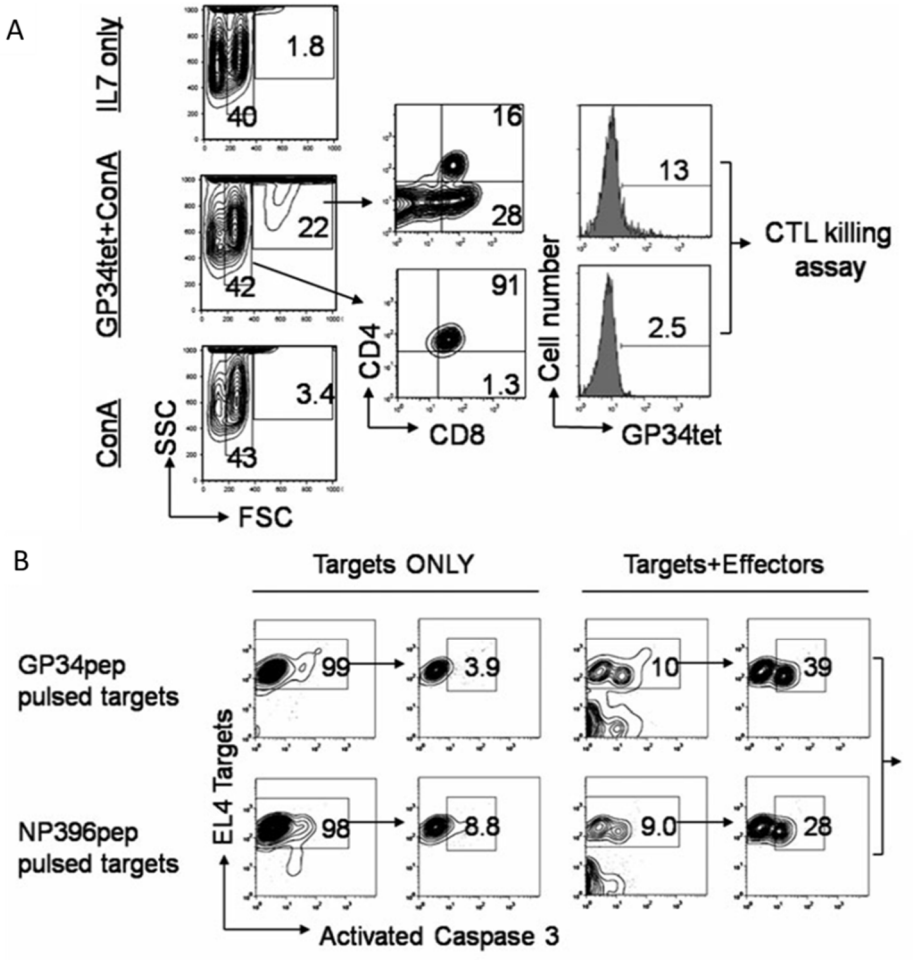

Figure 2.12: Antigen-specificity and functionality of *in vitro*-differentiated DP cells A) Antigen specific $CD8^+$ LCMV.GP34-specific T cells were generated in cultures that employed LCMV.GP34p-MHC Class I tetramers. B) LCMV.GP34-specific T cells exhibited specific cytotoxic activity against peptide-loaded target cells.[§§§]

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2.5 INFLUENCE OF MHC LIGAND DENSITY AND PRESENTATION ON TCR SIGNALING AND T CELL ACTIVATION

T cells are activated by engagement of their TCRs with pMHCs displayed on the surface of APCs. These interactions are essential for the recognition of foreign antigens in the periphery and are critical for the adaptive immune response. TCR-pMHC engagement results in the phosphorylation of the TCR-CD3 cytoplasmic domain by Src-family tyrosine kinases, followed by docking of adaptor proteins on these phosphorylated domains.[94] The result is a complex signaling unit consisting of TCR-CD3 cytoplasmic domains, adaptor proteins, and kinases.[94]

2.5.1 MHC/HLA Oligomer Technology

MHC tetramers consist of 4 peptide-loaded MHC molecules that are non-covalently bound by a streptavidin molecule. Tetramers that are tagged with fluorescent molecules allow enumeration or enrichment of antigen-specific T cell populations by flow cytometry.[33] This technology has been seminal in evaluating antigen-specific T cell responses such as proliferation, cytotoxic T cell assays, and cytokine production. In addition to detection, pMHC oligomers have been used for *in vitro* T cell expansion. It was recently demonstrated that aAPC microbeads presenting HLA-Ig dimers or HLA-tetramers are capable of activating and expanding antigen-specific $CD4^+$ and $CD8^+$ T cells.[29,30] In addition, stimulation of PBMCs with plate-bound tetrameric CMVp-MHCs resulted in significant expansion of CMV-specific $CD8^+$ T cells at levels comparable to that achieved with CMV peptide-loaded DCs.[31]

2.5.2 Density and Surface Presentation

The use of soluble crosslinking reagents, such as anti-TCR antibodies, has indicated that TCR oligomerization is a key step of activation. Several studies show that APCs pre-cluster their pMHCs prior to interaction with TCRs.[95] Activated B cells are

known to cluster peptide pMHCs in lipid rafts, and the disruption of lipid raft formation is deleterious to antigen presentation.[96] In addition, DCs have been shown to transport pMHC-rich endocytic vesicles that fuse to the plasma membrane, resulting in pMHC dense patches.[97] Indeed, it has been shown that monomeric pMHC molecules are not sufficient to stimulate or activate T cells.[98] Higher-valency oligomers such as MHC dimers, trimers, or tetramers more potently activate T cells due to increased binding avidity.[98-101]

Increasing evidence highlights the importance of pMHC surface presentation on inducing TCR signaling and directing T cell fate and effector function. Plate-immobilized peptide pMHC monomers, dimers, and tetramers can stimulate low-affinity TCRs significantly more effectively than their soluble counterparts.[102] Detailed studies have been done to control and characterize the role of ligand density on TCR signaling in mature T cells, as well as the effects of mobility, spatial distribution, and solid-phase presentation on T cell activation.[103] These studies are described in detail in Chapter 5. Much less is known about the influences of pMHC density and solid-phase presentation on immature thymocyte TCR signaling, maturation, and antigen-specific differentiation. In this treatise, the abilities of pMHC ligand density and surface presentation to differentially influence TCR stimulation and antigen-specific differentiation are explored.

2.6 SUMMARY AND CONCLUSION

The clinical promise of adoptive T cell transfer immunotherapy has been demonstrated with a wide variety of malignant and viral diseases. Despite its success, adoptive transfer is limited by inherent difficulties with isolating, expanding, and maintaining functional CTLs. Gene modification techniques, to express exogenous TCRs or CARs in endogenous hematopoietic cells, have been used to combat these problems, but pose their own set of complications including unforeseen specificities, insertional mutagenesis, and fatal systemic inflammatory responses. The results from numerous clinical studies suggest that a high-throughput method for generating functional, antigen-specific T cells from a renewable cell source, such as HSCs, is needed. By differentiating HSCs into antigen-specific T cells, the limitations and dangers of using patient-derived T cells and gene-modified T lymphocytes may be eliminated.

Based on the success of other groups at generating T cells from stem and progenitor cells *in vitro*, it was hypothesized that stem cells could be used as sources for adoptive transfer T cells. In this dissertation, a novel differentiation system, employing exogenous Notch and TCR ligands, was used to induce the generation of antigen-specific $CD8^+$ T cells from human cord blood $CD34^+$ HSCs. Following this, methods to enrich and expand progenitor-derived antigen-specific T cells were explored. Lastly, based on results that highlight the importance of TCR signaling strength on determining T cell fate, the effects of pMHC ligand density and solid-phase presentation on *in vitro* T cell development and antigen-specific differentiation were investigated.

2.7 ABBREVIATIONS

PTLD – post-transplant lymphoproliferative disease

CTL – cytotoxic T lymphocyte

TIL – tumor infiltrating lymphocyte

TCR – T cell receptor

CAR – chimeric antigen receptor

APC – antigen-presenting cell

FACS – fluorescence activated cell sorting

IL – interleukin

T_{REG} – regulatory T cell

PBMC – peripheral blood mononuclear cell

aAPC – artificial antigen-presenting cell

pHLA – peptide human leukocyte antigen

MART-1 – melanoma antigen recognized by T cells -1

gp – glycoprotein

CMV – cytomegalovirus

EBV – Epstein-Bar virus

IFNγ – interferon gamma

pMHC – peptide major histocompatibility complex

AIDS – acquired immunodeficiency syndrome

GVHD – graft-versus-host-disease

T_H – helper T cell

CEA – carcinoembryonic antigen

MAGE – melanoma-associated antigen

IRES – internal ribosome entry site

TAA – tumor-associated antigen

scFv – single chain variable fragment

ASK1 – apoptosis signal-regulating kinase 1

ATF2 – activating transcription factor 2

CAN – calcineurin

DAG – diacylglycerol

LAT – linker for activation of T cells

MMK – MAP kinase kinase

NFAT – nuclear factor of activated T cells

NF-κB – nuclear factor-κB

PIP3 – phosphatidylinositol-(3,4,5)-trisphosphate

PKCα – protein kinase Cα

PLCγ – phospholipase Cγ

SLP76 – SH2 domain-containing leukocyte protein of 76 kDa

ICOS – inducible T cell co-stimulator

TNFR – tumor necrosis factor receptor

MDSC – myeloid-derived suppressor cell

TRUCK – T cell redirected for universal cytokine-mediated killing

NK – natural killer

DC – dendritic cell

HSC – hematopoietic stem cell

CRS – cytokine release syndrome

T_N – naïve T cell

T_{CM} – central memory T cell

T_{EM} – effector memory T cell

TSP – thymus seeding prognitor

DN – double negative

DP – double positive

cTEC – cortical thymic epithelial cell

SP – single positive

mTEC – medullary thymic epithelial cell

TRA – tissue-restricted antigen

DLL – delta like ligand

DSL – Delta/Serrate/Lag

ADAM – a disintegrin and metalloproteinase

CBF1 – C-promoter binding factor 1

HES – hairy enhancer of split

ISP – intermediate single positive

FTOC – fetal thymus organ culture

RTOC – reaggregate thymus organ culture

ESC – embryonic stem cell

Flt3L – FMS-like tyrosine kinase 3 ligand

OP9-DL1 – OP9 cells expressing Delta like ligand 1

LSK – $Lin^{-}sca\text{-}1^{+}c\text{-}Kit^{+}$

RAG – recombination activating gene

LCMV – lymphocytic choriomeningitis virus

hTrp2 – human tyrosinase-related protein 2

MLR – mixed lymphocyte reaction

CFSE – carboxyfluorescein succinimidyl

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Chapter 3: Generating Functional, Human Antigen-Specific $CD8^+$ SP T Cells from $CD34^+$ Cord Blood-Derived Hematopoietic Stem Cells

[This chapter was adapted, with permission[****], from I. Fernandez, T.P. Ooi, and K. Roy[††††]. Generation of Functional, Antigen-Specific CD8+ Human T Cells from Cord Blood Stem Cells Using Exogenous Notch and Tetramer-TCR Signaling. Stem Cells. (2014) 32(1) 93-104. doi: 10.1002/stem.1512]

3.1 BACKGROUND AND MOTIVATION

T cells, unlike other lymphocyte subsets that mature in the bone marrow, develop exclusively in the thymus. It is well established that in addition to soluble cytokines, T lineage commitment is critically dependent on two temporally- and spatially-controlled signals provided by direct contact with thymic stromal cells: (i) Notch receptor-Delta like ligand (DLL) signaling and (ii) T cell receptor (TCR)-major histocompatibility complex (MHC)/human leukocyte antigen (HLA) signaling. Notch signaling is required for T lineage commitment and the development of double positive (DP) $CD4^+CD8^+$ early T cells.[1,2] DP cells undergo positive selection by interacting with antigen-loaded MHC/HLA molecules presented by cortical thymic epithelial cells. Specifically, cells with TCRs that are capable of interacting with self-antigen Class I or Class II MHC/HLA molecules escape apoptosis and differentiate into single positive (SP) $CD8^+CD4^-$ or $CD4^+CD8^-$ T cells, respectively. Subsequent negative selection in the thymic medulla

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eliminates SP T cells with TCRs that bind with high affinity to self-antigen MHC/HLA molecules, resulting in a diverse repertoire of mature, functional T cells.[1,2]

Fully mature T cells are characterized by the surface expression of an αβTCR heterodimer, which recognizes foreign-antigen MHC/HLA complexes on target cells. In particular, mature $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs), are responsible for eliminating pathogen-infected cells and tumor cells, thereby playing an essential role in immune function. This unique ability of $CD8^+$ T cells provides much promise for the field of cellular immunotherapy. The use of CTLs for autologous adoptive transfer has shown promise in treating many cancers including melanoma, renal cancer, leukemia, multiple myeloma, prostate cancer, Hodgkin's disease, and nasopharyngeal cancer, as well as post-transplant lymphoproliferative diseases (PTLDs).[3-9] Currently, T cells for such therapies are isolated from a patient's peripheral blood, expanded *ex vivo* for several weeks, and selected for antigen-specificity before being transplanted into the recipient.[3] Despite its immense clinical promise, adoptive T cell transfer is constrained by the difficulty and inefficiency of patient cell isolation, problems with expansion of primary cells *ex vivo*, a limited availability of HLA-matched donor cells, and the time required to process patient-isolated cells. Thus, scalable technologies for providing efficient, high throughput and readily available sources for antigen-specific, therapeutic T cells are needed.

In vitro T cell generation from stem cells has been explored extensively using co-culture with stromal cells known to support hematopoiesis. Retrovirally-transduced mouse bone marrow-derived stromal cells (OP9) that stably express the Notch ligands, DLL1 (OP9-DL1) or DLL4 (OP9-DL4), are capable of supporting the differentiation of mouse hematopoietic, embryonic, and induced pluripotent stem cells, as well as human hematopoietic stem cells (HSCs), into early T cells and $CD8^+$ SP T cells.[10-13] Recent

studies have also shown that plate-bound Notch ligands and a defined combination of soluble cytokines can induce early T cell development from mouse $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ (LSK) cells or human $CD34^{+}$ HSCs.[14-17] Taqvi et al. have previously shown that culturing mouse LSK cells with DLL4-functionalized microbeads in an insert co-culture system using OP9 cells can induce early T lineage commitment and differentiation without direct stromal cell contact.[18] However, generation of mature, functional antigen-specific SP T cells from these *in vitro* culture systems has not been reported extensively. Recently, antigen-specific T cells were expanded from a bulk population of OP9-DL1-derived murine $CD8^{+}$ SP T cells using bone marrow-derived dendritic cells (DCs) expressing various epitopes.[19] Lin et al. have also demonstrated the ability of antigen-loaded MHC Class I tetramers to generate, from mouse DP cells or embryonic stem cells (ESCs), a population of $CD8^{+}$ T cells specific for that particular antigen and capable of *in vitro* cytotoxic killing of target cells.[20] However, to date, direct *in vitro* generation of antigen-specific, functional human T cells from any stem cell population has not been achieved, except through stromal cell co-culture with HSCs retrovirally-transduced with specific TCRs.[21,22]

It was hypothesized that the thymic TCR-HLA interaction could be recreated *in vitro* using foreign antigen-loaded HLA tetramers to induce the differentiation of Notch-directed, human stem cell-derived early T cells into SP T cells specific for the same antigen. Indeed, by culturing human umbilical cord blood (UCB)-derived $CD34^{+}CD38^{-/low}$ HSCs with plate-immobilized DLL1, human HSCs can be directed into $CD1a^{+}CD7^{+}$ and $CD4^{+}CD8^{+}$ early T cells. Further culture with cytomegalovirus (CMV) or influenza (GIL) epitope-loaded HLA-A*0201 tetramers resulted in the generation of CMV-specific or GIL-specific $CD8^{+}$ T cells, respectively. These cells exhibited activation and *in vitro* cytotoxic functionality against peptide-loaded target cells as demonstrated by surface

presentation of the degranulation marker CD107a, production interferon gamma (IFNγ), and granzyme B secretion.

3.2 MATERIALS AND METHODS

3.2.1 Expansion of $CD34^+$ Cord Blood Cells

5 x 10^5 $CD34^+$ human cord blood mononuclear (CB-MN) cells (StemCell Technologies) were expanded in T25 tissue culture-treated flasks (Corning) using StemSpan® Serum Free Expansion Medium (StemCell Technologies) supplemented with the following human recombinant cytokines from Peprotech: FMS-related tyrosine kinase 3 ligand (Flt3L) (100 ng/mL), stem cell factor (SCF) (100 ng/mL), interleukin (IL)-3 (20 ng/mL), IL-6 (20 ng/mL), granulocyte-colony stimulating factor (G-CSF) (20 ng/mL), thrombopoietin (TPO) (50 ng/mL), and human low density lipoprotein (hLDL) (40 µg/mL) (StemCell Technologies). Cells were grown at 37°C and 5% CO_2. After 3 days, cells were transferred to T150 tissue culture treated flasks (Corning) and fresh media and cytokines were added to the cultures. Cells were expanded for a total of 7 days.

3.2.2 $CD34^+CD38^{-/low}$ Cell Sorting

Expanded CB-MN cells were collected and centrifuged at 300 x g for 5 minutes at 4°C. Cells were resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA). $CD34^+$ cells were enriched using MACS $CD34^+$ Microbead Kit and MS Columns (Miltenyi Biotec) according to the manufacturer's protocols. Resultant cells from CD34 positive selection were stained with PE-conjugated anti-CD34 monoclonal antibody (mAb) (Miltenyi Biotec, 130-081-002) and PECy7-conjugated anti-CD38 mAb (eBioscience, 25-0389-71). $CD34^+CD38^{-/low}$ cells were sorted by flow cytometry (BD FACSAria™ II, BD Biosciences) to obtain maximal purity.

3.2.3 Collection of OP9-DL1 Conditioned Medium

OP9-DL1 cells were kindly provided by Dr. Zúñiga-Pflücker (Toronto, Canada). 3.5 x 10^5 OP9-DL1 cells were seeded in T75 tissue culture-treated flasks and expanded in alpha minimum essential media (α-MEM) supplemented with 20% fetal bovine serum (FBS) (StemCell Technologies), sodium bicarbonate (2.2 g/L), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were grown at 37°C and 5% CO_2. Media was replaced when the cells were 80% confluent. For the next 3 days thereafter, OP9-DL1 conditioned media (CM) was collected, filtered through a 0.22 µm sterile membrane, and stored at 4°C.

3.2.4 Functionalization of Microplates with Notch Ligand Fc-DLL1

Protein A (10 µg/mL) (Sigma), diluted in PBS, was adsorbed on Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates (100 µL/well) for 30 minutes at 37°C and 5% CO_2. Wells were washed twice with PBS and blocked with HBSS/2% BSA at 25°C for 45 minutes. Residual blocking buffer was removed by washing twice with PBS. Human Fc-DLL1 (Enzo Life Sciences, 2.5 µg/mL or 5.0 µg/mL diluted in PBS), was incubated with Protein A-coated wells for 2 hours at 37°C and 5% CO_2. Wells were washed twice with PBS prior to cell seeding.

3.2.5 Notch Signaling-Induced T Cell Differentiation

Sorted $CD34^+CD38^{-/low}$ cells were seeded onto Fc-DLL1 coated plates at a density of 3.0 x 10^4 cells/well in OP9-DL1 CM supplemented with human recombinant Flt3L (10 ng/mL) and IL-7 (5.0 ng/mL) (Peprotech). After 13 days, all cells were collected and transferred to 24-well plates coated with Fc-DLL1 (5 x 10^5 cells/well). Cells were cultured with DLL1 plates for a total of 25 days. Media and cytokines were replenished every 3 days.

3.2.6 Antigen-Specific T Cell Differentiation

At day 25 of differentiation on DLL1-coated plates, CMVpp65 (NLVPMVATV) peptide (3 μg/mL), CMV (NLVPMVATV) HLA-A*0201 tetramers (3.0 μg/mL), or irrelevant control Influenza (GIL) (GILGFVFTL) tetramers (3.0 μg/mL) (Baylor College of Medicine) were added to the cultures in OP9-DL1 CM supplemented with human recombinant Flt3L (10 ng/mL), IL-7 (5.0 ng/mL), IL-2 (10 ng/mL) and co-stimulatory molecules anti-CD3 (1.0 μg/mL, Biolegend, 317304) and anti-CD28 (1.0 μg/mL, Biolegend, 302914). Cells were cultured with tetramers for a total of 7 days. Tetramers, peptide, media, cytokines, and co-stimulatory molecules were replenished every 3 days.

3.2.7 Flow Cytometry

At days 16, 22, and 25 of differentiation, cells were collected to quantify the expression of lymphoid and myeloid markers. Briefly, cells were harvested and resuspended in PBS containing 1% BSA and 2 mM EDTA. FcR blocking reagent (Miltenyi Biotec, 130-059-901) was added at a 1:100 dilution for 15 minutes at 4°C prior to staining. Cells were then labeled with a combination of mAbs against the lymphoid markers CD1a, CD7, CD4, CD8 and CD3 (CD1a-APC (BD Biosciences, 555807), CD7-PECy5 (BD Biosciences, 555362), CD4-PerCP (BD Biosciences, 550631), CD8-FITC (BD Biosciences, 555366), CD3-APC (BD Biosciences, 555335)) and myeloid markers CD11c, HLA-DR and HLA-ABC (CD11c-FITC (eBioscience, 11-0116-71), HLA-DR-APC (BD Biosciences, 559866;), HLA-ABC-PECy5 (eBioscience15-9983-41)) for 30 minutes at 4°C.

Seven days after adding HLA tetramers to the differentiation cultures (day 32), cells were harvested to determine the percentage of CMV-antigen specific $CD8^+$ cells in the cultures. Similarly, cells were stained with mAbs against CD8, CD3, CD4, and for antigen-specificity using APC-conjugated tetramers (CMVpp65 or GIL HLA-A*0201)

(Baylor College of Medicine Tetramer Production Facility). Isotype controls were included for each staining. To determine whether CMV-specific $CD8^+$ T cells generated from this system were monoclonal or polyclonal, staining was done at day 32 using IOTest® Beta Mark TCR Vβ Kit (Beckman Coulter). All samples were analyzed on a BD Accuri™ C6 Flow Cytometer (BD Biosciences) or BD FACSAria™ II (BD Biosciences). Data was analyzed using FlowJo Software (Tree Star, Inc.).

3.2.8 T Cell Functionality Assays: CD107a Mobilization and IFNγ Production

The functionality of antigen-specific T cells generated from $CD34^+CD38^{-/low}$ HSCs was assessed with a CD107a mobilization assay as previously described.[23] Differentiated cells were harvested and co-cultured with CMVpp65 peptide-loaded T2 target cells (174xCEM.T2; ATCC CRL-1992) in a CTL assay. All viable cells obtained from differentiation cultures were used as effector cells. T2 cells were loaded with 50 μg/mL of CMVpp65 peptide for 2 hours at 37°C in RPMI 1640 containing 10% human serum (Sigma Aldrich). Cells were washed twice with PBS to remove un-bound peptide. Incubation of effector and target cells was carried out for 5 hours at 37°C at a 5:1 effector to target (E:T) ratio. PE-conjugated anti-CD107a mAb (BD Biosciences, 555801) was added at the beginning of co-culture. Brefeldin A (BD Biosciences), a secretion inhibitor, was added after the first hour. Cells were collected and then surface-stained with FITC-conjugated anti-CD8 mAb and APC-conjugated tetramers (CMVpp65 or GIL HLA-A*0201) (Baylor College of Medicine). In some experiments, following surface staining, cells were fixed in Cytofix/Cytoperm solution (BD Biosciences) and stained with PECy7-conjugated anti-mouse IFNγ (BD Biosciences, 557844). Finally, cells were analyzed on a BD Accuri™ C6 Flow Cytometer (BD Biosciences). Data was analyzed using FlowJo Software (Tree Star, Inc.).

3.2.9 $CD8^+$ T Cell-Mediated Granzyme B Assay:

$CD8^+$ T cell-mediated granzyme B activity was analyzed using the GranToxiLux PLUS! Kit (OncoImmunin, Inc.). Briefly, target T2 cells were loaded with 50 µg/mL of CMVpp65 peptide for 2 hours at 37°C in RPMI 1640 containing 10% human serum. The fluorescent cell TFL2 dye (red) was added to the cells during the last 15 minutes of incubation. Cells were washed twice with PBS to remove un-bound peptide and TFL2 dye. Co-incubation of effector and target cells was carried out for 2 hours at 37°C at a 5:1 E:T ratio in the presence of the cell-permeable fluorescent (green) granzyme B substrate (OncoImmunin, Inc.). Cells were then collected and analyzed using FACSAria™ II (BD Biosciences). Data was analyzed using FlowJo Software (Tree Star, Inc.).

3.3 RESULTS

3.3.1 $CD1a^+CD7^+$ and $CD3^+CD4^+CD8^+$ Early T Cells can be Generated from *In Vitro* Expanded $CD34^+CD38^{-/low}$ HSCs Using Surface Immobilized Fc-DLL1 and OP9-DL1 Conditioned Media

The ability of surface-immobilized DLL1 and OP9-DL1 CM to induce T lineage commitment and early T cell development was evaluated. Human $CD34^+CD38^{-/low}$ UCB-derived HSCs were cultured on DLL1-functionalized plates in the presence of stromal cell-secreted factors (Figure 3.1). Briefly, $CD34^+$ UCB cells were expanded for 7 days. $CD34^+$ cells were enriched using magnetic microbeads and $CD34^+CD38^{-/low}$ cells were sorted to > 95% purity (Figure 3.1). Purified $CD34^+CD38^{-/low}$ cells were then plated on non-tissue culture-treated wells containing 2.5 μg/mL or 5 μg/mL of immobilized Fc-DLL1 supplemented with Flt3L and IL-7.

By day 8, most of the $CD34^+CD38^{-/low}$ cells differentiated into $CD34^-CD38^+$ hematopoietic cells, independent of Notch-induced signaling (results not shown). There was a modest population of $CD34^+CD38^{-/low}$ cells in all culture conditions (approximately 5.0-7.0%) that remained $CD34^+$ and gained CD38 ($CD34^+CD38^+$); however, by day 16 $CD34^+CD38^{-/low}$ cells from the Notch-induced cultures (either 2.5 or 5.0 μg/mL Fc-DLL1) rapidly differentiated into $CD34^-CD38^+$ cells compared to no ligand control cultures, which contained a higher percentage of $CD34^+CD38^+$ cells (Figure 3.2A). These results indicate that there was more rapid induction of hematopoiesis in the presence of Notch ligands, consistent with previous results from other groups.[14-17] By day 25, the majority of cells from Fc-DLL1 and no DLL1 conditions were $CD34^-CD38^+$ (Figure 3.3A).

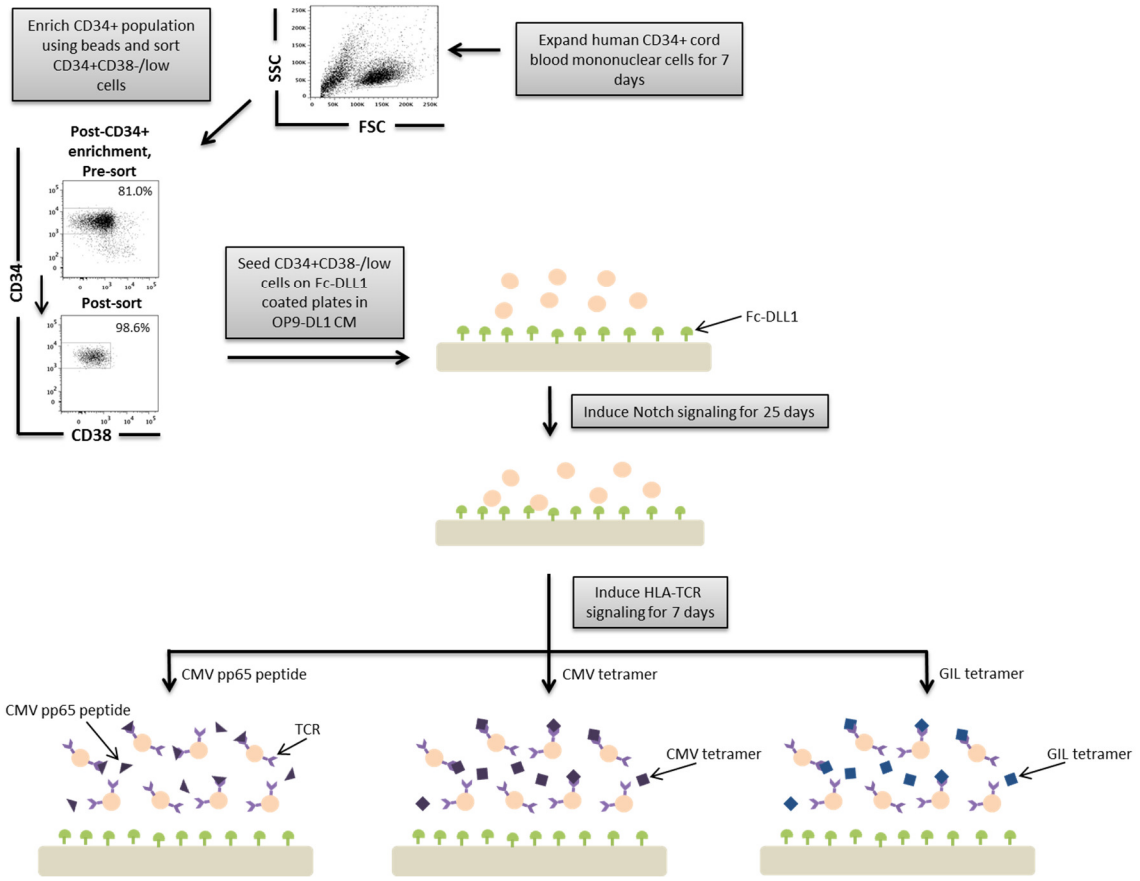


Figure 3.1: Schematic for the generation of antigen-specific $CD8^+$ T cells from cord blood $CD34^+$ HSCs. $CD34^+$ cord blood cells were expanded for 7 days. The $CD34^+CD38^{-/low}$ HSC population was enriched, sorted, and seeded onto DLL1-coated plates. HLA-TCR signaling was induced using CMVpp65 peptide, CMV epitope-loaded HLA-A*0201 Class I tetramers, or GIL epitope-loaded HLA-A*0201 Class I tetramers.

At days 16, 22, and 25, the ability immobilized Notch ligands to direct T lineage differentiation and generate early T lymphocytes was evaluated. Cultures were analyzed for the presence of $CD1a^+CD7^+$ cells, previously identified as early T cells, and $CD4^+CD8^+$ DP T cells (Figure 3.2B, 3.2C, and Figure 3.3B, 3.3C).[10,12] At day 16, conditions employing Notch ligands (2.5 µg/mL Fc-DLL1) had about twice as many $CD1a^+CD7^+$ cells compared to the no ligand control (Figure 3.2B). After 25 days of

culture with Fc-DLL1, the cultures had about 10 times as many $CD7^+CD1a^+$ cells compared to the control (Figure 3.3B). Furthermore, it was observed that a significantly higher percentage of CD7+ lymphoid-committed cells were generated in Notch ligand cultures compared to the control at both time points. As expected, the $CD7^+$ population was much higher in peripheral blood mononuclear cells (PBMCs) (Figure 3.3B).

To investigate whether more mature T cells were present in the culture, cells were analyzed for the expression of CD4 and CD8 (Figure 3.2C and Figure 3.3C). At day 22, conditions employing Fc-DLL1 had twice as many $CD4^+CD8^+$ cells compared to the no ligand control, indicating progression through the double negative stages to the DP stage of T cell development (Figure 3.2C). By day 25, four times as many $CD4^+CD8^+$ early T cells were found in the DLL1 cultures compared to the control (Figure 3.3C). At day 22, a slightly higher percentage of $CD4^+CD8^-$ cells were observed in the no ligand control cultures compared to Notch ligand cultures (Figure 3.2C). By day 25, the percentage of $CD4^+CD8^-$ cells in the Notch ligand culture was similar to that of the no ligand control (Figure 3.3C). It was hypothesized that $CD4^+CD8^-$ cells differentiated due to the presence of HLA Class II-presenting myeloid cells. This hypothesis was confirmed by the observation of $CD11c^+$HLA-DR^+ cells in all conditions (Figure 3.4).

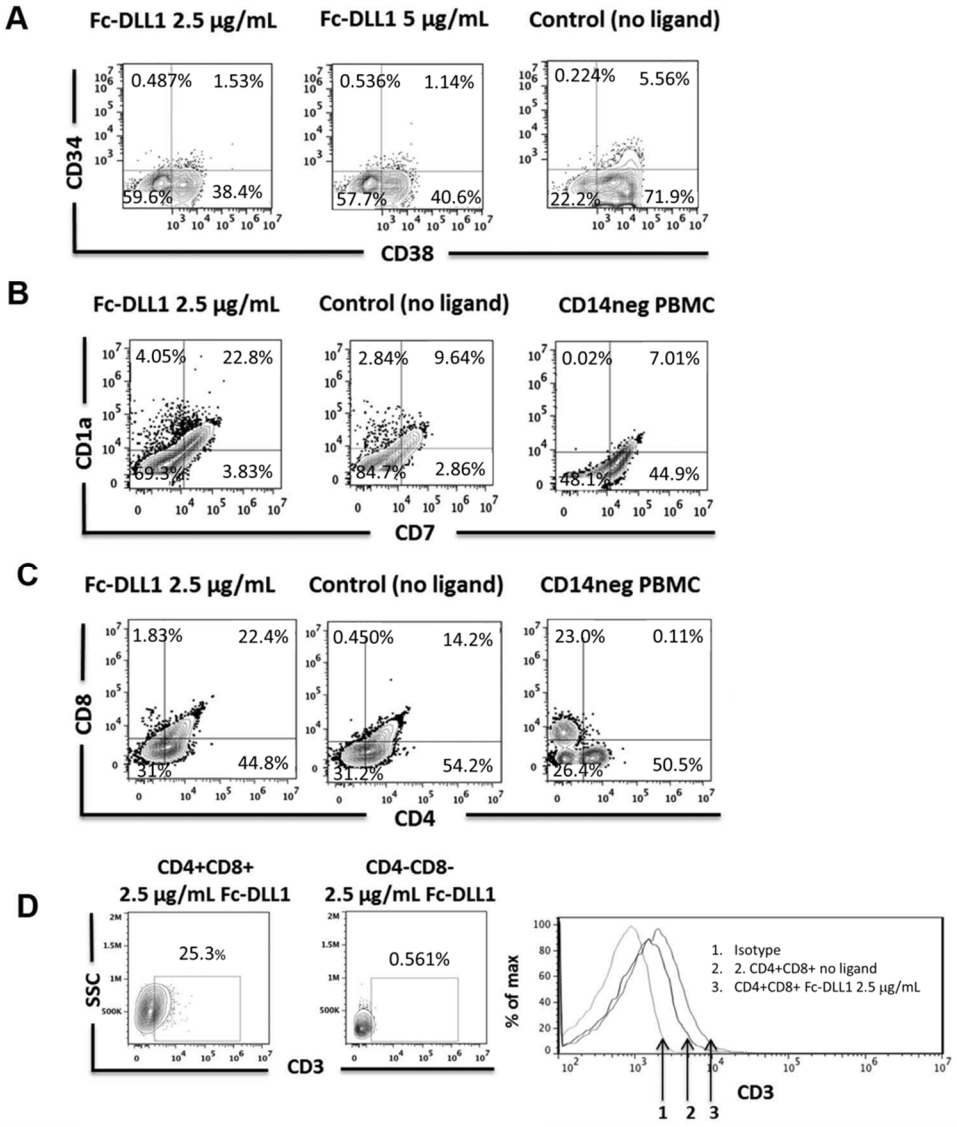
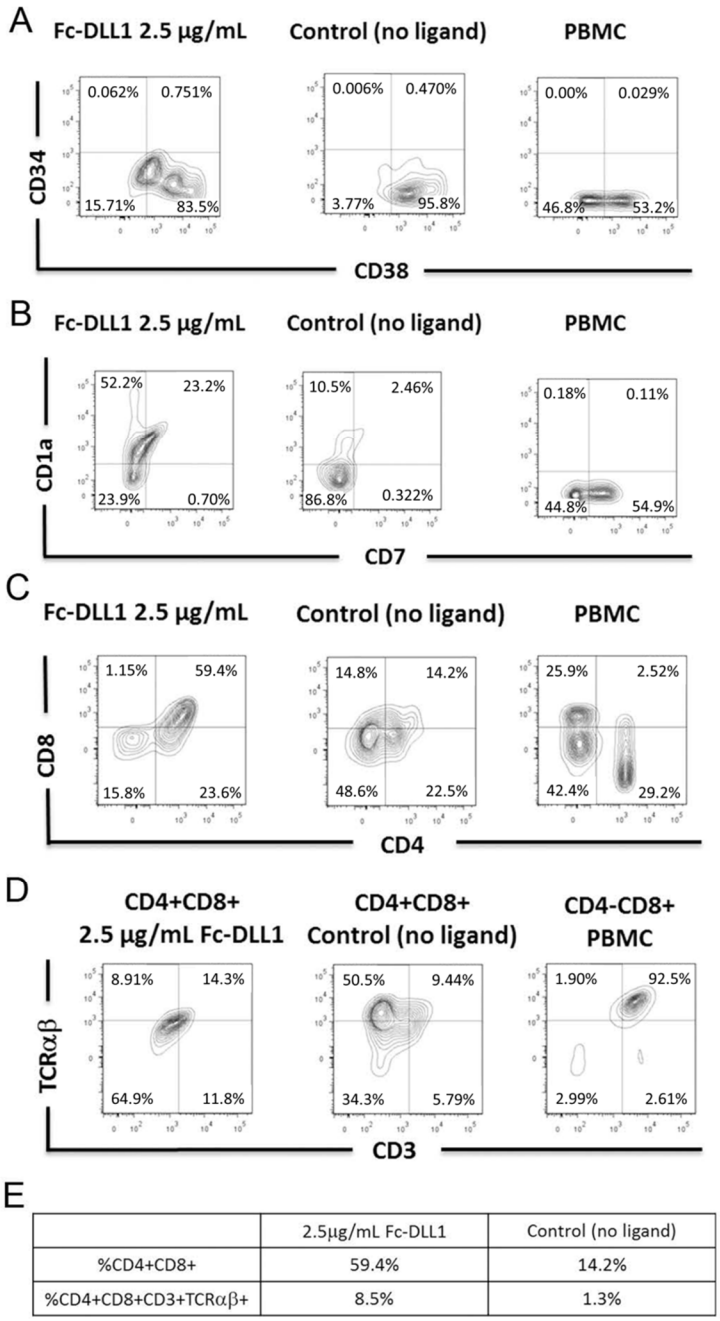


Figure 3.2: Day 16 and day 22 characterization of cells derived from $CD34^{+}CD38^{-/low}$ cells in Notch signaling-induced differentiation cultures. Flow cytometry analysis of A) CD34 and CD38 and B) CD1a and CD7 expression at day 16 and C) CD4 and CD8 and D) CD3 expression at day 22.

In order to gauge T cell differentiation in the presence of immobilized DLL1, CD3 and TCRαβ expression was evaluated. Although $CD4^+CD8^+$ populations were present in all conditions at day 22, DP cells from the no ligand cultures expressed lower levels of CD3 compared to cells differentiated with Fc-DLL1 (Figure 3.2D). It was hypothesized that the DP cells from the the no ligand control differentiated due to the presence of Notch ligands on other cells within the culture. Indeed, it has been shown by Karanu, et al. that human $CD34^+CD38^-$ cells, myeloid cells, and mature $CD3^+$ T cells express DLL1 and/or DLL4.[24] At day 25, approximately 10-15% of the $CD4^+CD8^+$ DP cells generated from DLL1 and control cultures expressed CD3 and TCRαβ (Figure 3.3D and 3.3E). Out of the starting population, 8.5% of the cells differentiated with Notch ligand were $CD4^+CD8^+CD3^+TCR\alpha\beta^+$, compared to only 1.3% from the no ligand control. The expression of CD3 and TCRαβ on $CD8^+CD4^-$ PBMCs are shown as staining controls. As expected the majority of the $CD8^+CD4^-$ PBMCs express CD3 and TCRαβ, as these cells are mature T cells (Figure 3.3D and 3.3E).



E

	2.5µg/mL Fc-DLL1	Control (no ligand)
%CD4+CD8+	59.4%	14.2%
%CD4+CD8+CD3+TCRαβ+	8.5%	1.3%

Figure 3.3: Day 25 characterization of cells derived from $CD34^{+}CD38^{-/low}$ cells in Notch signaling-induced differentiation cultures. Flow cytometry analysis of A) CD34 and CD38, B) CD1a and CD7, C) CD4 and CD8 expression, and D) CD3 and TCRαβ expression. E) Table indicating the percentage of the total population that is $CD4^{+}CD8^{+}$ or $CD4^{+}CD8^{+}CD3^{+}TCR\alpha\beta^{+}$.

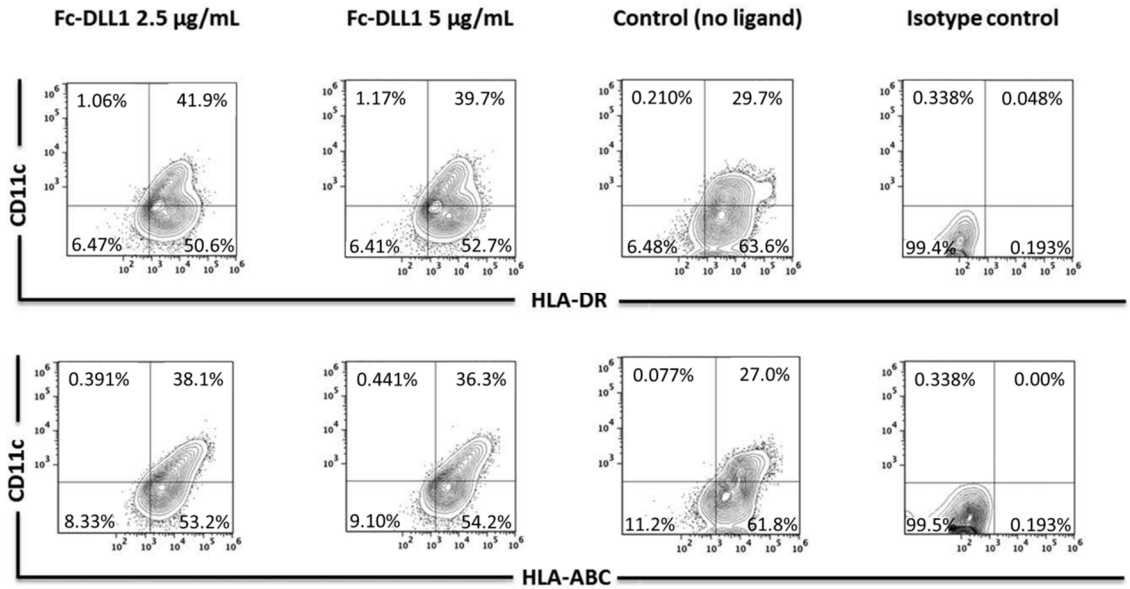


Figure 3.4: Day 16 characterization of myeloid cells derived from $CD34^{+}CD38^{-/low}$ cells in Notch signaling-induced differentiation cultures. Flow cytometry analysis of CD11c, HLA-ABC and HLA-DR expression.

3.3.2 Peptide-Loaded HLA-A*0201 Tetramers Induce the Generation of Antigen-Specific $CD8^{+}$ T Cells

On day 25, 3.0 µg/mL of CMVpp65 peptide, CMVpp65 HLA-A*0201 Class I tetramers, or GIL HLA-A*0201 Class I tetramers were added to the culture along with exogenous co-stimulatory molecules, anti-CD3 and anti-CD28 (Figure 3.5A). Seven days after peptide or tetramer addition, cells were analyzed for antigen-specificity and functionality. After 7 days of culture with peptides or tetramers, $CD8^{+}$ cells were found to be primarily $CD4^{-}$, and a low percentage of $CD4^{+}CD8^{+}$ cells was observed (Figure 3.6). It was hypothesized that the population of $CD4^{+}CD8^{-}$ cells found in the culture differentiated due to the presence of HLA Class II-presenting myeloid cells (Figure 3.4).

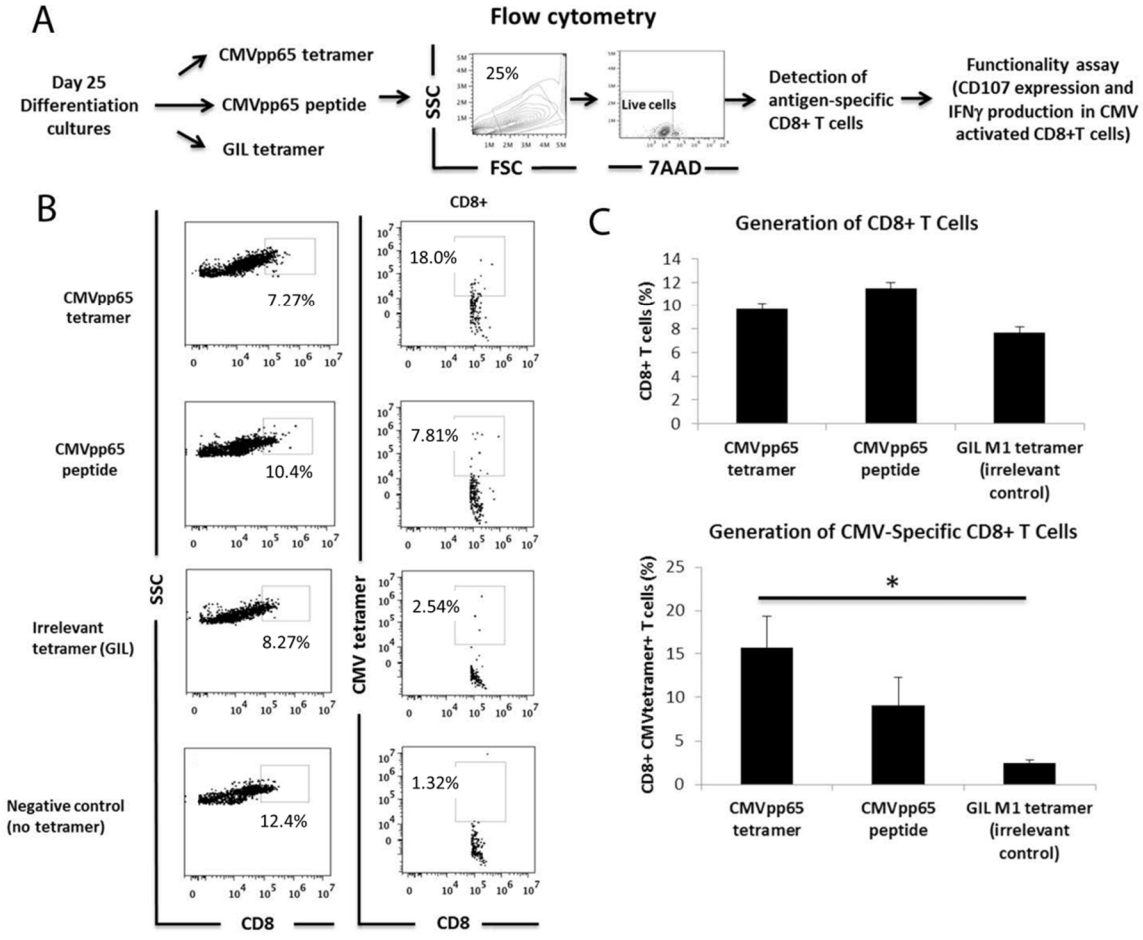


Figure 3.5: Generation of CMV antigen-specific $CD8^+$ T cells in the presence of CMVpp65 tetramer or peptide. A) Experimental design. B) CMV antigen-specific $CD8^+$ T cells were generated by adding CMVpp65 tetramers, CMVpp65 peptide, or an irrelevant control tetramers (GIL tetramer) at day 25 of differentiation. C) Bar graphs represent percentage of $CD8^+$ T cells (gated on total cells) and percentage of $CD8^+CMV^+$ cells (gated on $CD8^+$ cells) (n = 5, $*P < 0.05$).

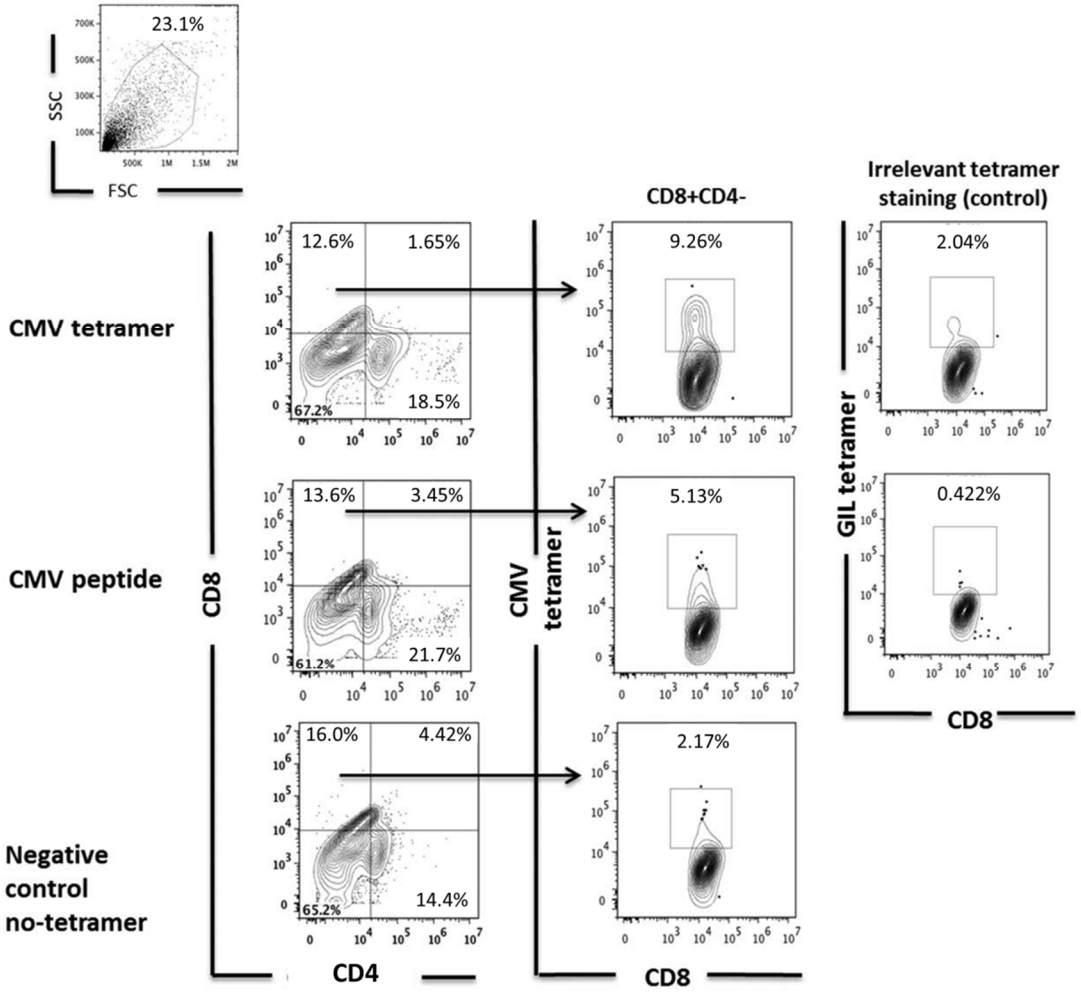


Figure 3.6: $CD8^+$ T cells generated with CMVpp65 tetramer or peptide are predominantly $CD4^-$. CMV-specific T cells are $CD8^+CD4^-$, and a small population of $CD4^+CD8^+$ cells remains after culture with tetramers or peptide. $CD4^+CD8^-$ cells may have differentiated due to the presence of Class II-presenting cells in culture.

Of the cells differentiated with CMV tetramer or CMVpp65 peptide, approximately 5.64%-26.90% were found to be $CD8^+$ T cells. Of the $CD8^+$ T cells, 2.91%-21.60% were CMV-specific. Cells differentiated with CMVpp65 peptide or tetramer consistently had higher percentages of CMV-specific $CD8^+$ T cells compared to the cells cultured with the GIL irrelevant control tetramer (Figure 3.5B, 3.5C). These

results were consistent over 5 independent samples (Figure 3.5C). Similarly, GIL-specific $CD8^+$ T cells could be generated by incorporating GIL tetramers into the differentiation cultures (Figure 3.7). These results indicate that for the generation of antigen-specific T cells, the described system is not restricted to a specific tetramer. Interestingly, incubation of differentiating cells with CMVpp65 peptide also initiated antigen-specific T cell development (Figure 3.5B). This may be attributed to the presence of DCs, as well as presentation of the epitope via HLA Class I molecules expressed by nucleated cells in the differentiation cultures (Figure 3.4).

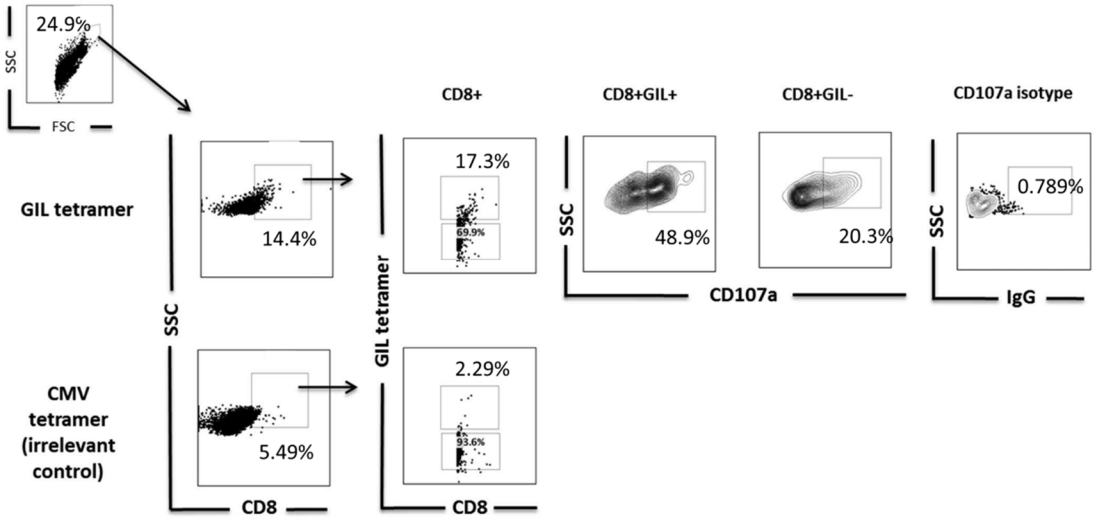


Figure 3.7: Generation of Influenza antigen-specific $CD8^+$ T cells in the presence of Influenza GIL tetramer. $CD8^+GIL^+$ cells were generated when GIL HLA-A*0201 tetramers were added to differentiation cultures for 7 days. 48.9% of these cells expressed CD107a when cultured with GIL epitope-loaded target cells at an effector to target ratio of 5:1.

3.3.3 Human HSC-Derived Antigen-Specific $CD8^+$ T Cells are Functional

Functionality of CMV-specific $CD8^+$ T lymphocytes was determined by CD107a surface mobilization, intracellular IFNγ production, and granzyme B-mediated cytotoxicity in the presence of antigen-loaded target cells.[23,25-27] During the process of

cell killing, $CD8^+$ effector T cells release cytotoxic mediators such as perforins and granzymes upon antigen engagement. CD107a, also known as lysosomal-associated membrane protein-1, is a vesicle membrane protein that becomes transiently mobilized to the cell surface during degranulation, the initial event that takes place during target cell lysis. Upon co-culture of differentiated effector T cells with peptide-loaded target cells, CD107a mobilization was quantified. Additionally, expression of IFNγ, a critical cytokine that is produced by $CD8^+$ T cells upon antigen recognition, was evaluated by intracellular staining. Lastly, granzyme B activity in target cells was measured, as this provides a quantitative assessment of T cell-mediated cellular cytotoxicity.[28]

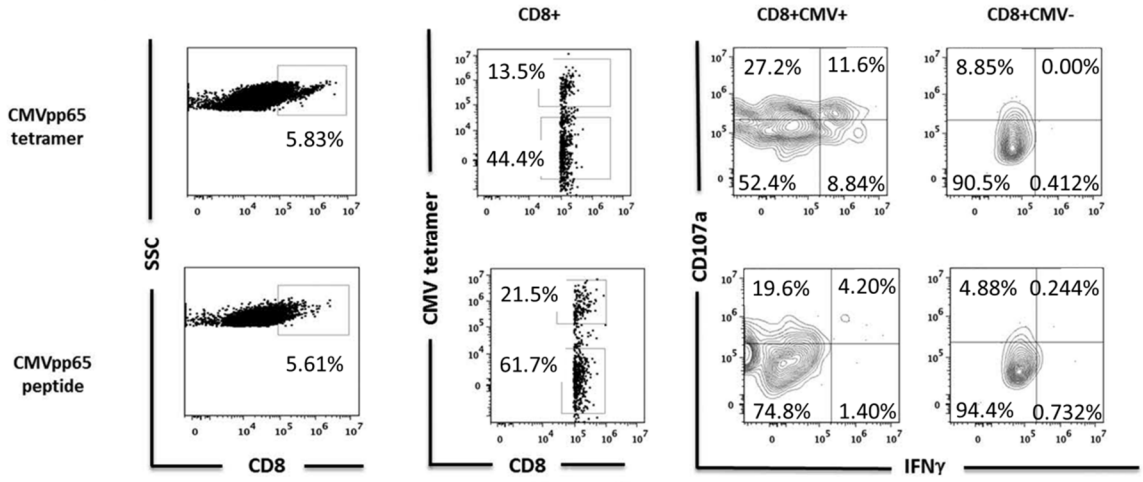


Figure 3.8: CMV antigen-specific $CD8^+$ T cells express CD107a and produce IFNγ after culture with epitope-presenting target cells.

For the functional assays, all cells from differentiation cultures were harvested and used as effector cells. T2 target cells were loaded with CMVpp65 peptide for 2 hours. For CD107a/IFNγ assays, effectors and peptide-loaded target cells were co-cultured for 5 hours at an E:T ratio of 5:1, in the presence of Brefeldin A and anti-CD107a mAb. For granzyme B assays, effectors and peptide-loaded targets were co-cultured for 2 hours in the presence of a fluorescent granzyme B substrate. Following

stimulation, the ability of CMV-specific cells to degranulate and produce IFNγ was examined (Figure 3.8).

Approximately 13% of $CD8^+$ T cells generated from CMV tetramer differentiation cultures in this experiment were CMV-specific (Figure 3.8, top panel). After stimulation with target cells, about 40% of the tetramer-differentiated CMV^+ cells expressed CD107a. From those, 11.6% produced IFNγ (Figure 3.8, top panel). Similarly, $CD8^+CMV^+$ cells were generated from CMVpp65 peptide differentiation cultures (Figure 3.8, bottom panel). About 25% of those cells expressed CD107a upon co-culture with target cells, but less than 5% produced IFNγ. Although stimulation with CMVpp65 peptide for 7 days resulted in a high number of antigen-specific $CD8^+$ T cells, peptide-stimulation may not be sufficient to induce CTL effector functionality. It is possible that more than 7 days of peptide stimulation is needed for activation.

The expression of CD107a between $CD8^+CMV^+$ and $CD8^+CMV^-$ cells generated from CMV tetramer cultures and CMV peptide cultures was compared (Figure 3.8). Although CMV-specific CD107a surface expression was higher in $CD8^+CMV^+$ T cells than in $CD8^+CMV^-$ T cells, $CD8^+CMV^-$ cells still expressed CD107a non-specifically. This could be due to several confounding factors. First, due to cell number limitations, all cells from the differentiation cultures were used as effector cells. In addition, the use of co-stimulatory molecules anti-CD3 and anti-CD28, as well as IL-2, to stimulate T cell proliferation may induce a background level of activation, resulting in CD107a expression. It is also possible that some of the non-CMV-specific $CD8^+$ T cells recognized self-antigens presented by T2 cells.

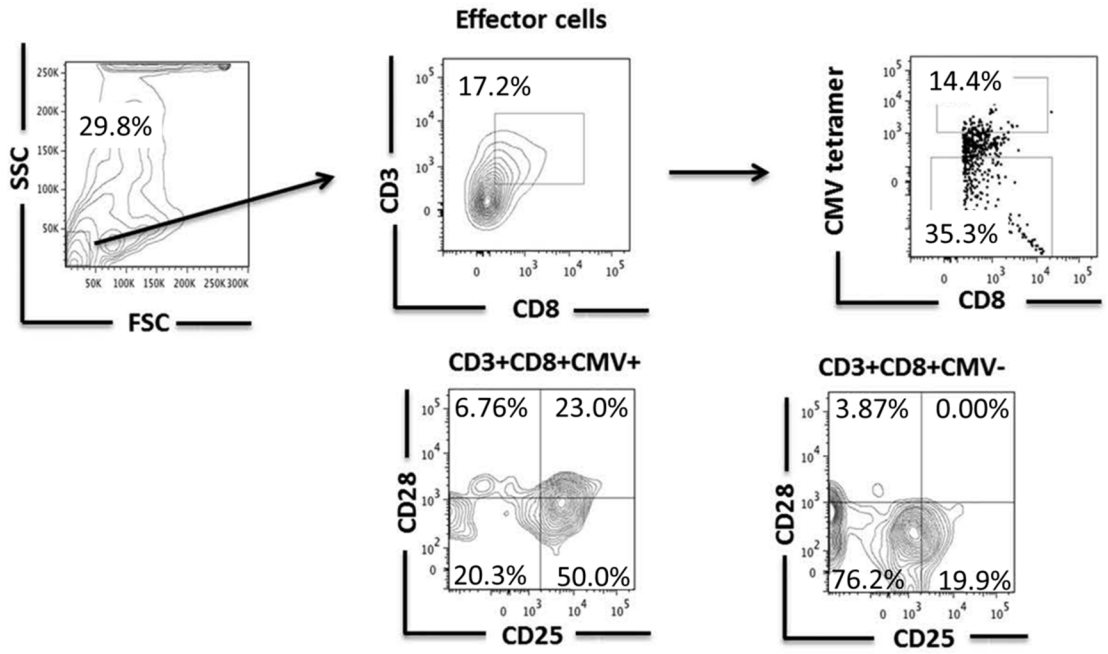


Figure 3.9: Tetramer-differentiated CMV-specific $CD8^+$ T cells express activation markers CD25 and CD28.

The expression of CD28 and CD25, a co-stimulation and activation marker relevant in the cytotoxic process initiated by $CD8^+$ T cells, on the antigen-specific *in vitro*-generated T cells was determined. The results (Figure 3.9) show that approximately 30% of the $CD3^+CD8^+CMV^+$ *in vitro*-differentiated T cells were $CD28^+$ and the majority expressed CD25. In contrast, most of the $CD3^+CD8^+CMV^-$ *in vitro*-differentiated T cells were CD28- and a lower percentage expressed CD25.

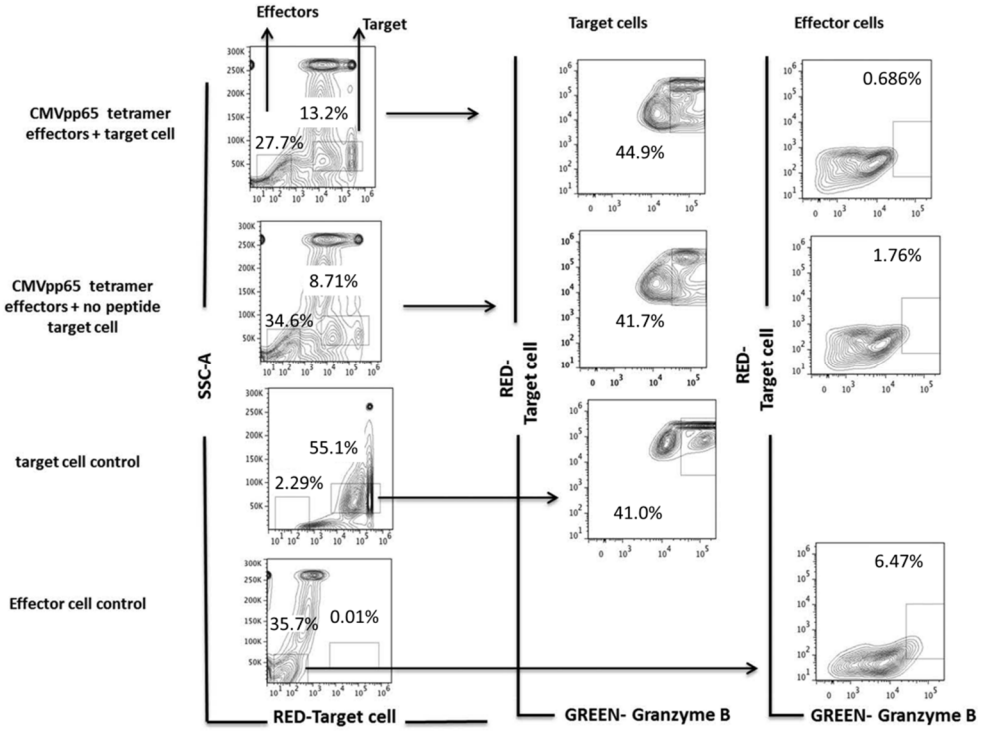


Figure 3.10: CMV antigen-specific $CD8^+$ T cells facilitated granzyme B-mediated killing. CMVpp65 peptide-loaded targets were labeled with a red dye and incubated, in the presence of granzyme B substrate (green), with CMVpp65 tetramer-differentiated cells (effector cells) at a 5:1 E:T ratio for 2 hours. No peptide-loaded target cells, target cells alone, and effector cells alone are shown as controls. Target cells were gated on FSC^{hi} cells and effector cells were gated on FSC^{low} cells.

Cytotoxic $CD8^+$ T cell-mediated killing of target cells was evaluated by measuring granzyme B activity inside target cells (Figure 3.10). T2 target cells were fluorescently labeled and then co-incubated with CMVpp65 tetramer-differentiated cells in the presence of a fluorescent granzyme B substrate. Cleavage of the substrate results in increased green fluorescence in target cells. Granzyme B activity was detected in about 44.9% of the CMVpp65 peptide-loaded target cells after incubation with effector cells containing CMV antigen-specific $CD8^+$ T cells (Figure 3.10, upper panel), compared to 41.7% and 41% background levels detected in no peptide-loaded target cells and no

effector conditions, respectively (Figure, 3.10 middle panels). There did not appear to be significant differences in the percentages of granzyme B^+ targets in the different conditions, but the data demonstrate that a significant proportion of the *ex vivo* differentiated $CD8^+$ cells exhibit effector function. Staining of effector cells alone was evaluated as a control. As expected there was no significant detection of granzyme B, as it exists in an inactive form in effector cells (Figure 3.10, right panel). The relatively high level of background staining may be attributed to prolonged culture with the tetramers and co-stimulatory molecules, as well as possible cross-reactivity of effector T cells. The presence of dead target cells at beginning of the CTL experiment, prior to incubation of targets with effectors, may have also contributed to the high background.

3.3.4 Antigen-Specific $CD8^+$ T Cells are Polyclonal

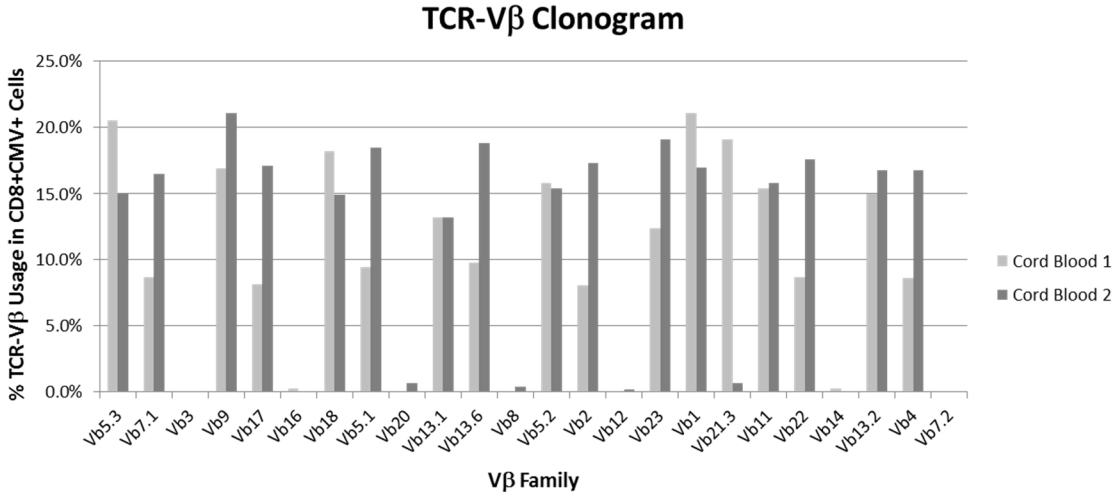


Figure 3.11: Clonogram showing TCR Vβ repertoire of CMV antigen-specific $CD8^+$ T cells after differentiation with CMVpp65 tetramers for 7 days.

To provide insight about the clonality of the CMV-specific $CD8^+$ T cells generated from differentiation cultures, TCR Vβ analysis via flow cytometry was performed. Interestingly, multiple Vβ families were represented in the CMV-specific

$CD8^+$ T cell population (Figure 3.11). This finding was consistent over two independent sets of differentiated CB-MN cells.

3.4 DISCUSSION

The ability of HSCs to differentiate into various blood lineages, as well as their unique capacity to self-renew, makes them an attractive source from which therapeutic T cells can be generated.[29,30] HSCs are isolated from the bone marrow, peripheral blood, or umbilical cord blood (UCB) and can be expanded, thereby providing a large, readily-available cell source that could be differentiated into lineage-specific therapeutic cells as needed. In addition, problems associated with allogeneic T cell transfer, such as the shortage of donors and the difficulty of finding HLA-matched donor cells, may be overcome through the use of HSCs as a source for adoptive transfer T cells.[31] In particular, human umbilical cord and placenta, which support the developing fetus during pregnancy, are delivered with the baby and can be easily retrieved after birth. Numerous public cord blood banks have been developed worldwide to collect, type, and cryopreserve cord blood for potential future transplant.[32] The development of an efficient and scalable differentiation system that could generate antigen-specific, functional T cells from cord blood HSCs could enable important therapeutic opportunities in a variety of diseases.

With advancements in *in vitro* stem cell differentiation technologies, stem cell-derived therapies are becoming more of a reality. To specifically induce T lymphoid lineage commitment, ESCs or HSCs can be co-cultured with OP9-DL1 cells, bone marrow-derived stromal expressing the Notch ligand, DLL1.[10-13] The OP9-DL1 co-culture system is now an established method for generating early T cells and $CD8^+$ SP T cells; however, the directed differentiation of human antigen-specific T cells, without retroviral transduction of specific TCRs, has not been reported.[10-13,21,22,33] Furthermore, large scale production of therapeutic T cells for eventual clinical application via co-culture with genetically modified cells is difficult, both from pharmaceutical and

regulatory perspectives. Therefore, in order for *in vitro* T cell differentiation to become clinically applicable, a system that is capable of producing antigen-specific T cells on a large scale, without relying on complicated co-culture methods or genetic modification, is necessary.

The development of a stromal cell contact-free system that facilitates the differentiation of human cord blood-derived HSCs into functional, antigen-specific T cells was described in this chapter. Notch signaling for the generation of early T cells was mediated by plate-immobilized Notch ligands in the presence of soluble factors from stromal cell CM. Recent studies have indicated the importance of Notch ligand endocytosis in Notch signaling and T cell development.[34,35] In the context of OP9-DL1 or OP9-DL4 co-culture, mutants that are unable to undergo Notch ligand endocytosis or recycling have a reduced capacity to induce Notch signaling and support T cell development.[34,35] While the system detailed in this chapter utilizes plate-immobilized Notch ligands that are unable to be endocytosed, the development of $CD1a^+CD7^+$ and $CD4^+CD8^+$ early human T cells was still observed. This finding is consistent with that of other groups utilizing immobilized Notch ligands for HSC expansion.[13-17] The frequency of T cell progenitors may increase by using Notch ligand-presenting cells; however, the goal here is to develop a stromal cell contact-free system for inducing T cell development that can be easily scaled up and is clinically translatable.

TCR signaling, induced by antigen-loaded HLA-A*0201 Class I tetramers, provided sufficient signals to generate a population of polyclonal, antigen-specific $CD8^+$ T cells. The results demonstrate that the antigen-specific T cells developed from this differentiation system exhibited effector function against peptide-loaded target cells, as indicated by the surface presentation of activated cytotoxic-specific marker, CD107a, production of intracellular IFNγ, and granzyme B activity, albeit with some non-

specificity and high background killing. For $CD8^+$ T cell immunotherapy to be successful, efficient T cell activation and cytotoxic activity in response to foreign antigen is crucial. While the affinities of the T cells generated through the described system may vary depending on the antigen, the continued presence of HLA Class I tetramers, and the exogenous use of anti-CD28 and IL-2 may prime and increase the functional avidity of the *in vitro*-derived antigen-specific $CD8^+$ T cells.[36-39] Furthermore, antigen-specific T cells could be sorted from the bulk population and expanded with several rounds of tetramer stimulation to increase specificity. Thus, the described system is a promising platform for the *in vitro* generation of cytotoxic T cells with therapeutic efficacy.

The TCRVβ repertoire of the antigen-specific $CD8^+$ T cells was evaluated in order to elucidate how TCR-MHC/HLA signaling, induced by antigen-loaded HLA tetramers, facilitates the development of antigen-specific T cells *in vitro*. It was hypothesized that two possible mechanisms contributed to the *in vitro* generation of antigen-specific T cells (Figure 3.12): (1) Proliferation of a single random antigen-specific $CD8^+$ T cell clone or (2) TCR gene rearrangement induced by co-stimulatory molecules and/or soluble factors within the differentiation system that increase the probability of generating antigen-specific T cells, which are further expanded by the continued presence of tetramers and cytokines.

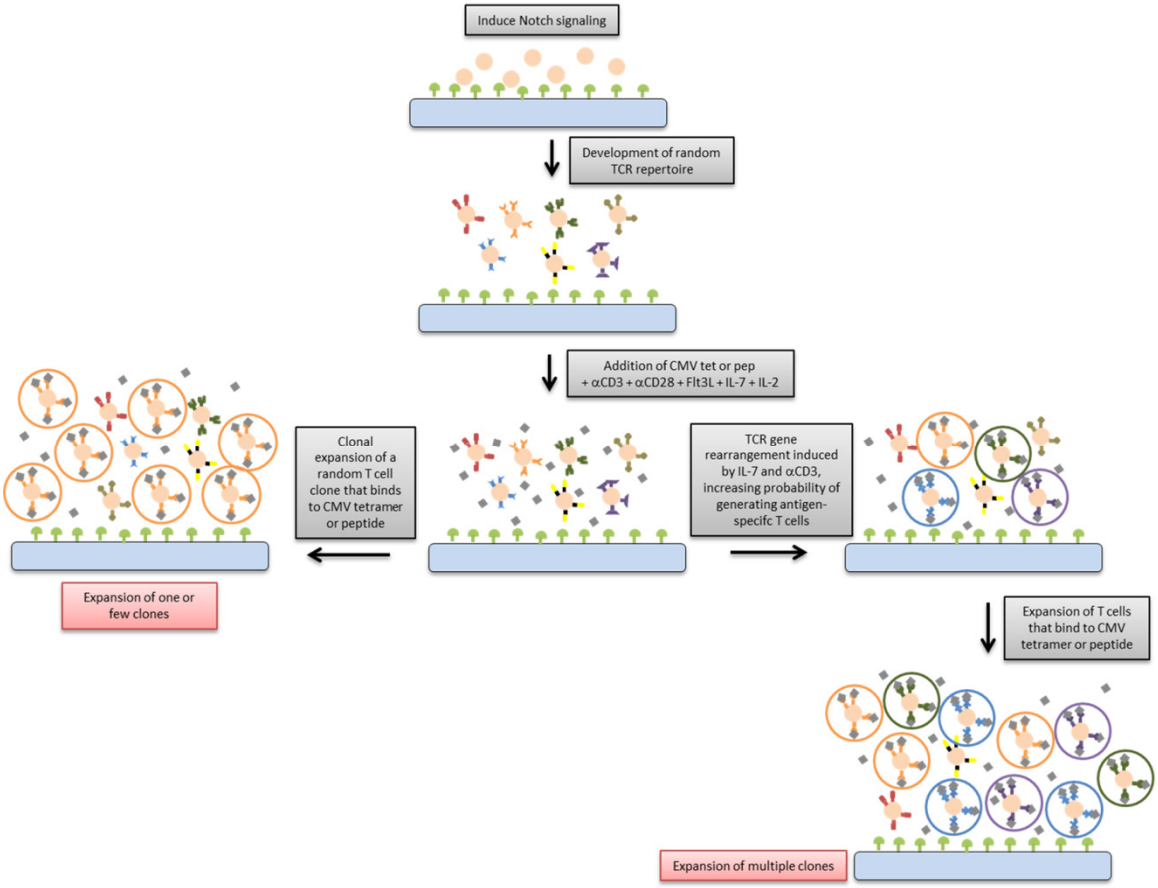


Figure 3.12: Schematic overview of how tetramer-differentiated, antigen-specific T cells are being generated *in vitro*.

Recent studies indicate that *in vitro* T cell differentiation systems using OP9-DL1 co-cultures are capable of producing populations of antigen-specific $CD8^+$ T cells that can be expanded by epitope-presenting DCs.[19] Since CMV or GIL epitope-loaded HLA tetramers were added to the all differentiated cells, it is possible that randomly generated T cell clones, specific for either antigen, are expanded. If this were the case, the clonal diversity of antigen-specific cells would be low. The data indicate that several unique Vβ families were highly represented in the antigen-specific T cell repertoire, suggesting that

the primary mechanism of tetramer-mediated generation of antigen-specific T cells may not be limited solely to expansion of a single or few clones.

Given the diverse clonality of CMV-specific T cells generated from the differentiation system, it was hypothesized that another, or an additional mechanism, may be responsible for the generation of antigen-specific T cells. In the periphery, if a T cell cannot recognize a presented antigen, it can either undergo anergy and death or TCR revision, which may allow the cell to respond to an antigen.[40] During TCR revision, T cells lose surface expression of the TCR, upregulate recombinase machinery, and rearrange genes to produce an extrathymically generated TCR that is then expressed on the cell surface.[41-43] This phenomenon was first identified in Vβ5 transgenic mice that lost Vβ5 expression and gained an endogenous Vβ repertoire similar to that of wild type mice as they aged.[44] $V\beta5^{low}$ peripheral T cells were found to have downregulated surface TCR expression and high levels of RAG1, RAG2, and TdT indicating gene rearrangement of the TCR loci.[44]

Recently, it was demonstrated that treatment of mouse DP thymocytes with antigen-loaded $H\text{-}2K^b$ MHC Class I tetramers, anti-CD28, anti-CD3, IL-2, and IL-7 results in RAG-1 upregulation, suggesting that TCR gene rearrangement is initiated or continued due to the presence of MHC tetramers.[20] Furthermore, an *in vitro* model of TCR revision in mature human $CD8^+$ T cells, induced by the presence of anti-CD3 and IL-7, was recently described.[45] IL-7 has been shown to induce T cell survival and facilitate chromatin remodeling at the TCR loci in developing, immature T cells.[46-48] Thus, due to the presence of HLA-A*0201 Class I tetramers and other soluble stimulatory factors within the culture, the possibility that TCR gene rearrangement may be occurring *in vitro* cannot be eliminated. While more in-depth studies on clonality are

required to confirm this hypothesis, the reported findings present important insights into the mechanism behind *in vitro* T cell development.

One concern with this *in vitro* differentiation system is that while it provides a means for selection of T cells specific for one antigen, there is no mechanism that ensures negative selection to eliminate self-reactive cells. In addition, the data indicates that CMV-specific T cells displayed only slightly more cytotoxic activity against CMVpp65 peptide-displaying target cells compared to target cells loaded with no peptide. It is hypothesized that a pure, antigen-specific $CD8^+$ T cell population could be easily sorted at the end of the differentiation to increase specificity and reduce the possibility of recovering self-reactive T cells. To increase the yield of the desired antigen-specific $CD8^+$ T cells, several rounds of tetramer stimulation could be done by continuously pulsing the cells with Class I tetramers and co-stimulatory molecules, anti-CD3, anti-CD28, and IL-2. It has been shown that bead-based artificial antigen-presenting cells, displaying pMHC tetramers and co-stimulatory molecules, anti-CD3, anti-CD28, and 4-1BB, can activate and expand antigen-specific T cells.[49] Furthermore, Savage et al. have shown that soluble Class I MHC tetramers can be used to expand $CD8^+$ T cells *in vitro*.[50] Given the previous success of these groups, it is speculated that the antigen-specific $CD8^+$ T cells generated from the described *in vitro* differentiation system can be expanded to yield therapeutic numbers.

3.5 CONCLUSIONS

In this chapter, a novel, *in vitro* system which can be used to generate antigen-specific T cells from human hematopoietic progenitors, was detailed. By utilizing plate-immobilized and soluble ligands, the need for feeder cells and retroviral transfection was eliminated, thereby providing a practical and scalable technology that could facilitate the high-throughput production of therapeutic T cells from hematopoietic stem cells.

3.6 ABBREVIATIONS

MHC – major histocompatibility complex

DLL – Delta like ligand

HLA – human leukocyte antigen

TCR – T cell receptor

DP – double positive

SP – single positive

CTL – cytotoxic T lymphocyte

PTLD – post-transplant lymphoproliferative disease

OP9 – bone marrow derived stromal cell

OP9-DL1 – bone marrow derived stromal cell expressing delta like 1

OP9-DL4 – bone marrow derived stromal cell expressing delta like 4

HSC – hematopoietic stem cell

LSK – $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$

DC – dendritc cell

ESC – embryonic stem cell

UCB – umbilical cord blood

CMV – cytomegalovirus

GIL – influenza

CB-MN – cord blood mononuclear cell

Flt3L – FMS-related tyrosine kinase 3 ligand

SCF – stem cell factor

IL - interleukin

G-CSF – granulocyte colony-stimulating factor

TPO – thrombopoietin

hLDL – human low-density lipoprotein

PBS – phosphate buffered saline

BSA – bovine serum albumin

EDTA – ethylenediaminetetraacetic acid

mAb – monoclonal antibody

PE – phycoerythrin

Cy – cyanine

a-MEM – alpha minimum essential media

FBS – fetal bovine serum

FcR – Fc receptor

APC – allophycocyanin

FITC – fluorescein isothiocyanate

PBMC – peripheral blood mononuclear cell

NLVPMVATV – (asparagine – leucine – valine – proline – methionine – valine – alanine – threonine – valine)

GILGFVFTL – (glycine – isoleucine – leucine – glycine – phenylalanine – valine – phenylalanine – threonine – leucine)

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Chapter 4: Enrichment and Expansion of Progenitor-Derived Antigen-Specific $CD8^+$ T Cells for Adoptive Transfer Immunotherapy

4.1 INTRODUCTION

In the previous chapter, a novel method to differentiate human $CD34^+$ cord blood hematopoietic stem cells (HSCs) into functional, antigen-specific $CD8^+$ T cells was presented. While a population of antigen-specific T cells was generated using this system, the resulting efficiency and cell yield were low. Approximately 0.5-1.0% of the starting $CD34^+$ population differentiated into functional, antigen-specific $CD8^+$ T cells. During clinical adoptive transfer, 10^8-10^{12} CTLs are needed for each infusion in order to ensure an effective response against the tumor or viral load, and a pure, antigen-specific T cell population is needed to minimize graft-versus-host response. Background killing levels of differentiated T cells in cytotoxic T lymphocyte (CTLs) assays were high, possibly due to the use of bulk mixed populations as effectors. To address these issues, it was hypothesized that the enrichment and expansion of stem cell-derived or progenitor-derived antigen-specific $CD8^+$ T cells would yield a pure population of antigen-specific T cells in clinically relevant numbers. In this chapter, the enrichment and expansion of progenitor-derived antigen-specific $CD8^+$ T cells, using Class I tetramers, co-stimulatory molecules, and proliferative cytokines is described.

4.1.1 Antigen-Presenting Cells for T Cell Activation and Expansion

Classically, T cell immunotherapy involves the *ex vivo* stimulation and expansion of antigen-specific T cells using professional antigen-presenting cells (APCs), in particular dendritic cells (DCs), which are equipped with the machinery to activate naïve T cells. APCs provide three signals that induce T cell activation and proliferation:[1-3]

- Signal 1: Antigen presentation
- Signal 2: Co-stimulation

- Signal 3: Cytokine release

The first signal, antigen recognition, occurs through the interaction of a T lymphocyte's T cell receptor (TCR) with a peptide major histocompatibility complex (pMHC in mice, peptide human leukocyte antigen (pHLA) in humans) presented by professional APCs, which express MHC Class I and Class II molecules.[1-3] Class I molecules present antigens derived from intracellular proteins and engage $CD8^+$ T cells, while Class II molecules present extracellular peptides and interact with the TCRs of $CD4^+$ T cells.[1-3] Once $CD8^+$ T cells are activated, they become CTLs and can induce antigen-specific cell lysis.[1-3] Activated $CD4^+$ cells, or T helper (T_H) cells, release cytokines to stimulate CTL activation and B cell-dependent antibody production.[1-3] TCR ligation can also be induced using agonistic antibodies against CD3, which trigger the TCR-CD3 signaling complex, resulting in the transduction of signals required for T lymphocyte activation.[4,5]

Signal 2, or co-stimulation, is an antigen-independent signal needed for full T cell activation.[1-3] Upon encounter with infection or cellular damage, APCs up-regulate molecules B7-1 (CD80) and B7-2 (CD86) which interact with CD28 on the T cell surface to induce full activation.[1-3] Interaction of CD80 or CD86 with inhibitory molecule, cytotoxic T lymphocyte associated protein 4 (CTLA-4), can have immunosuppressive effects on T cell function and differentiation.[1-3]

Lastly, cytokines released by APCs or $CD4^+$ T_H cells, or in an autocrine fashion by CTLs, further direct effector differentiation and function by binding to cytokine receptors.[1-3] These cytokines are essential for the survival, proliferation, and function of $CD8^+$ T cells. In particular, interleukin (IL)-2 is produced by T cells upon initial contact with the APC and promotes expansion and effector cell differentiation.[1-3] Activating cytokines secreted by APCs such as IL-12, IL-15, IL-21, and type I interferons (IFNα/β)

are necessary for proper T cell function and crucial for T effector differentiation. Inhibitory cytokines such as IL-4, IL-5, and IL-10 are known to reduce the immune response and can induce tolerance.[1-3]

The use of APCs, such as DCs, for *ex vivo* T cell expansion is constrained by the inherent disadvantages of using primary cells. The isolation of autologous DCs is often inefficient and time-consuming, rendering the procedure impractical for patients with advanced stages of disease.[6] The quality of patient-derived autologous DCs can be variable due to sub-optimal antigen loading and the immunosuppressive tumor microenvironment.[6] The reagents required for stimulation of DCs are also expensive. To overcome the difficulties with using autologous DCs, artificial antigen-presenting cells (aAPCs) that display antigens and/or co-stimulatory molecules in a controlled manner, have been produced or fabricated.[7] To date, numerous groups have successfully used aAPCs to expand adoptive transfer tumor- or virus-specific T cells *in vitro*.

4.1.2 T Cell Expansion Using Cell-Based aAPCs

4.1.2.1 Insect Cells

Insect cells were first used to investigate the molecular mechanisms of antigen processing and presentation. *D. melanogaster* cells lack the Transporter Associated with Antigen Presentation 1 (TAP1) and TAP2 peptide transporters needed to load endogenous peptides on nascent MHC Class I molecules, and are therefore good candidates with which to study exogenously loaded epitopes.[8] Cai et al. found that *D. melanogaster* cells, transfected with murine Class I molecules alone, were not able to stimulate naïve T cells.[9,10] However, co-transfection of B7-1 and intracellular adhesion molecule 1 (ICAM-1) turned Class I-expressing *D. melanogaster* cells into potent APCs

with the ability to induce naïve transgenic 2C $CD8^+$ T cell proliferation, IL-2 production, and CTL generation.[9,10]

In a phase I adoptive transfer clinical trial, *D. melanogaster* aAPCs expressing HLA-A2.1, CD80, and I-CAM were loaded with the HLA.A2.1-restricted tyrosinase 369-377 peptide and used to expand antigen-specific CTLs from patient-isolated $CD8^+$ T cells; subsequent re-stimulation was done using autologous peripheral blood mononuclear cells (PBMCs) and supplemental IL-2 and IL-7. After 1 month, 10^9 CTLS were generated, with 10-30% being specific for the melanocyte antigen.[11] The problem with using *D. melanogaster* cells for applications requiring extended culture is that they are optimally viable at 27°C.[11] When cultured at 37°C, *D. melanogaster* cells experience a drastic loss in viability by 12 hours. Cell death results in a reduced number of TCR-pMHC contacts and a release of antigens from dying cells. Therefore, despite being seminal tools for studying antigen-presentation, *D. melanogaster* cells are not practical aAPCs for clinical applications.

4.1.2.2 Murine Fibroblasts

Murine fibroblasts were used in experiments to investigate the role of co-stimulatory molecules directly involved in murine or human CTL activation. Latcouhe et al. co-transfected mouse fibroblasts with genes encoding B7.1, ICAM-1, leukocyte function antigen (LFA)-3, a single pHLA Class I complex, and human β_2-microglobulin.[12] They found that these aAPCs elicited robust stimulation and expansion of the antigen-specific human CTLs when they presented individual epitopes derived from influenza matrix, melanoma antigen recognized by T cells-1 (MART-1) or glycoprotein (gp)100 proteins.[12] In subsequent experiments, Papanicolaou and colleagues demonstrated that murine HLA-A2.1 aAPCs expressing the cytomegalovirus (CMV)

P495 epitope or full-length pp65 protein rapidly stimulated and expanded P495-specific CTLs as effectively as autologous PBMCs.[13]

4.1.2.3 Human Erythroleukemia Cells

June and colleagues recently engineered an aAPC system for rapid, polyclonal expansion of human CTLs. The K562 erythromyeloid cell line was stably transfected to express human Fcγ receptor (CD32), and human 4-1BB co-stimulatory molecules. These cells, named K32/4-1BB, could then be coated with anti-CD3 and anti-CD28 (K32/4-1BB/CD3/28) and used to non-specifically expand CTLs.[14] It was found that K32/4-1BB/CD3/28 cells could induce reproducible, long-term proliferation with an average of 410-fold higher T cell numbers compared to anti-CD3/28 beads.[14] K32/4-1BB/CD3/28 cells were also able to expand MHC tetramer-sorted primary influenza matrix protein (MP) peptide-specific $CD8^+$ T lymphocytes for up to 60 days, without loss of cytotoxicity for flu MP peptide-pulsed target cells.[14] Similar studies indicate that K32/4-1BB/CD3/28 cells can support the long-term expansion of $CD4^+$ T cells.[15]

4.1.3 T Cell Expansion Using Acellular aAPCs

Cellular APCs have provided much insight about the requirements for T cell activation and expansion; however, the use of cell-based APCs is not ideal or economically viable for clinical applications. The lack of reproducibility and consistency of cell-derived APCs has warranted the need for "off the shelf" acellular APCs that can be made with Good Manufacturing Practices. Additionally, such acellular systems eliminate the need for allogeneic culture or the use of virally infected cells. As a result, much work has been done to fabricate and study how acellular aAPCs can be used to induce $CD8^+$ and $CD4^+$ T cell expansion.

4.1.3.1 Anti-CD3/Anti-CD28 Microbeads

The use of magnetic microbeads for *ex vivo* T cell expansion was initially pioneered by Levine and colleagues. They demonstrated that beads, covalently modified with anti-CD3 and anti-CD28, could exponentially expand $CD4^+$ T cells for over 60 days without the use of additional feeder cells. This expansion was enhanced by the addition of exogenous IL-2.[16] Later studies have shown that $CD8^+$ T cells can also be expanded using anti-CD3/anti-CD28 beads, albeit with a lower proliferative capacity and greater levels of apoptosis, than $CD4^+$ cells.[17] Anti-CD3/anti-CD28 beads are commercially available,[5] and have been shown to expand CD4+ and CD8+ T cells to levels comparable to the "Rapid Expansion Protocol" (REP), which utilizes soluble anti-CD3 and mononuclear cells.[18]

4.1.3.2 MHC/HLA Multimers

Oelke et al. have fabricated aAPC microbeads with immobilized pHLA.A2-Ig dimers and anti-CD28 antibodies.[19-21] Microbeads presenting pHLA.A2-Igs loaded with high-affinity CMVpp65 peptide or low-affinity MART-1 peptide were able to induce robust expansion of the respective antigen-specific CTLs. Cells cultured with these beads expanded 10^6-fold after two months and maintained antigen-specificity of greater than 85%.[19-21] Antigen-specific human $CD4^+$ T cell expansion has also been achieved using microbeads, conjugated with pHLA tetramers and anti-CD28 molecules, as aAPCs.[22] In addition to bead-immobilized Class I molecules, soluble Class I MHC tetramers have shown success in expanding $CD8^+$ CTLs *in vitro*.[23] Savage and colleagues have shown that cytotoxic T cells recognizing HLA-2/Melan-A and HLA-A2/Gag complexes could be expanded 100-fold over the 3 weeks with tetramer stimulation.[23]

4.2 OBJECTIVE

In this chapter, the enrichment and expansion procedures used to generate clinically relevant numbers of progenitor-derived antigen-specific $CD8^+$ T cells are presented. While work described in the previous chapter was done using human stem cells, this chapter focuses on the use of mouse T cell progenitors as the starting cell source. Because murine thymocytes are easily retrievable and readily available in large quantities, they were useful tools for these proof-of-concept experiments. It was hypothesized that robust expansion of progenitor-derived antigen-specific $CD8^+$ single positive (SP) T cells could be initiated by mimicking the three signals provided by APCs: i.) antigen-presentation, ii.) co-stimulation, and iii.) proliferative cytokines.

Based on the success of other groups in expanding antigen-specific CTLs using MHC multimers, it was hypothesized that signal 1, antigen-presentation, could be induced using soluble, cognate pMHC Class I tetramers. Anti-CD3 antibodies, which can trigger the TCR signaling pathway, were included to enhance stimulation.[19,21,23] The second signal, co-stimulation, was provided by incorporating anti-CD28 antibodies into the expansion media. Lastly, the proliferation-inducing cytokine, IL-2, was used to induce the third signal needed for T cell expansion. It was hypothesized that cognate Class I tetramers, together with anti-CD3, anti-CD28, and IL-2, would act synergistically to induce the robust expansion of progenitor-derived antigen-specific $CD8^+$ SP T cells. A schematic of the experimental procedure can be found below (Figure 4.1). Briefly, double positive (DP) thymocytes from C57BL/6 mice were sorted and cultured with ovalbumin (OVA)257p- or lymphocytic choriomeningitis virus (LCMV).GP34p-MHC Class I tetramers, anti-CD3, anti-CD28, IL-2, and IL-7 for 7 days. Antigen-specific $CD8^+$ SP T cells were then sorted and expanded using the respective Class I tetramers, anti-CD3,

anti-CD28, and IL-2 for an additional 7 days. Following expansion, CTL functionality was determined via granzyme B activity assays (Figure 4.1).

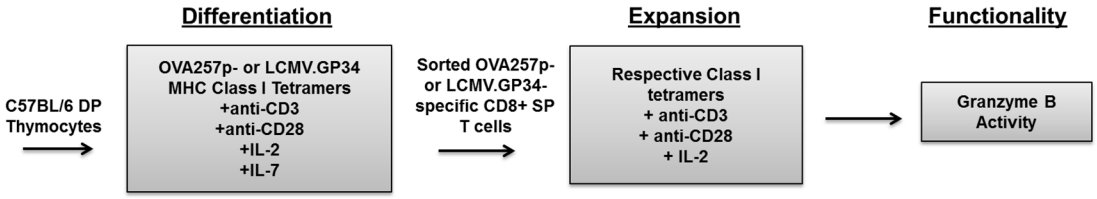

Figure 4.1: Experimental design. Sorted $CD4^+CD8^+$ DP thymocytes from C57BL/6 mice were differentiated with OVA257p- or LCMV.GP34p-MHC Class I tetramers, anti-CD3, anti-CD28, IL-2, and IL-7 for 7 days. OVA257- or LCMV.GP34-specific $CD8^+$ T cells were sorted and expanded for 7 days using the respective Class I tetramers, anti-CD3, anti-CD28, and IL-2. CTL functionality was determined via granzyme B activity.

4.3 MATERIALS AND METHODS

4.3.1 Thymocyte Isolation

Thymi from C57BL/6 mice (The Jackson Laboratory) were isolated. Thymi were ground on top of BD Falcon Blue Nylon Mesh 40 μm Cell Strainers (BD Biosciences, 352340) to release thymocytes into a single cell suspension. Red blood cells were lysed using 1X RBC Lysis Buffer (eBioscience, 00-4333-57) at a concentration of 2 mL/thymus for 15 minutes at 20°C (room temperature). Cells were counted and washed with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 300 x g for 5 minutes at 4°C. Thymocytes were resuspended at a concentration of 10^7 cells/mL in PBS containing 0.1% BSA and 2 mM EDTA and processed for cell sorting.

4.3.2 Double Positive Thymocyte Sorting

Thymocytes were treated with FcR Block (eBioscience, 16-0161) at a 1:100 dilution for 15 minutes on ice, then labeled with FITC-conjugated anti-CD8a (clone 53-6.7, eBioscience, 11-0081) and PECy7-conjugated anti-CD4 (clone RM5-4, eBioscience, 25-0042) at a 1:100 dilution for 30 minutes on ice. Cells were then washed with 10-fold excess of PBS containing 0.1% BSA and 2 mM EDTA and centrifuged at 300 x g for 5 minutes at 4°C. Pellets were resuspended at 10^7 cells/mL in PBS containing 0.1% BSA and 2 mM EDTA and transferred to sterile polystyrene 12 x 75 mm test tubes. $CD4^+CD8^+$ DP thymocytes were sorted using FACSAriaIIIu (BD Biosciences) and FACSDiva Software (BD Biosciences).

4.3.3 Antigen-Specific $CD8^+$ T Cell Differentiation Using OVA257p-MHC or LCMV.GP34p-MHC Class I Tetramers

Sorted C57BL/6 DP thymocytes were seeded in Corning® Not Treated Tissue Culture clear, flat-bottom 12-well plates with OVA257p-MHC (10 μg/mL) or LCMV.GP34p-MHC tetramers (10 μg/mL, Baylor College of Medicine Tetramer Production Facility) at a concentration of 10^6 cells/well in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). All wells were supplemented with anti-CD28 (1.0 μg/mL, clone 37.51, eBioscience, 16-0281), anti-CD3 (1.0 μg/mL, clone 17A2, eBioscience, 16-0032), IL-2 (10 ng/mL), and IL-7 (10 ng/mL) at a 1.0 mL working volume. Cells were cultured for a total of 7 days. Media and supplemental factors were replenished every 3 days.

4.3.4 OVA257 and LCMV.GP34 Antigen-Specific $CD8^+$ T Cell Sorting

Differentiated cells were collected and washed with a 10-fold excess of PBS containing 0.1% BSA and 2mM EDTA to remove excess media. Cells were counted using trypan blue exclusion and pelleted at 300 x g for 5 minutes at 4°C. The cell pellet was resuspended in PBS containing 0.1% BSA and 2 mM EDTA using gentle vortexing at a concentration of 10^7 cells/100 μL. Following resuspension, the cells were stained with APC-conjugated LCMV.GP34p- or OVA257p-MHC tetramers at a 1:100 dilution on ice for 30-45 minutes. The cells were then treated with FcR Block (eBioscience, 16-0161) at a 1:100 dilution for 15 minutes on ice, then labeled with FITC-conjugated anti-CD8a (clone 53-6.7, eBioscience, 11-0081) and PE-conjugated or PECy7-conjugated anti-CD4 (clone RM5-4, eBioscience, 12-0042 or 25-0042, respectively) at a 1:100 dilution for 30 minutes on ice. Cells were then washed with 10-fold excess of PBS containing 0.1% BSA and 2 mM EDTA and centrifuged at 300 x g for 5 minutes at 4°C. Cell pellets were resuspended at 10^7 cells/mL in PBS containing 0.1% BSA and 2 mM EDTA and

transferred to sterile polystyrene 12 x 75 mm test tubes at a concentration of 10^6 – 10^7 cells/100 µL. Tetramer-specific $CD4^-CD8^+$ SP thymocytes were sorted using FACSAriaIIIu (BD Biosciences) and FACSDiva Software (BD Biosciences).

4.3.5 Expansion of OVA257- or LCMV.GP34-specific $CD8^+$ SP T cells Using OVA257p-MHC or LCMV.GP34p-MHC Class I Tetramers

The sorted OVA257- or LCMV.GP34-specific $CD8^+$ SP T cells were cultured for 7 days in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), anti-CD28 (1.0 µg/mL, clone 37.51, eBioscience, 16-0281), anti-CD3 (1.0 µg/mL, clone 17A2, eBioscience, 16-0032), IL-2 (10 ng/mL), and OVA257p- (10 µg/mL) or LCMV.GP34p-MHC tetramers (10 µg/mL), respectively, in Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates at 200 µL working volumes. Seeding density ranged from 30,000 – 100,000 cells/well. Media and supplemental factors were replenished every 3-4 days.

4.3.6 Characterization of T Cells after 7 Days of Expansion with OVA257p-MHC or LCMV.GP34p-MHC Class I Tetramers

After expansion, cells were counted and characterized by flow cytometry to confirm enrichment and expansion. Cells were collected in polystyrene 12 x 75 mm test tubes and washed with 10-fold excess of PBS containing 0.1% BSA and 2 mM EDTA. The cell pellet was resuspended in PBS containing 0.1% BSA and 2 mM EDTA using gentle vortexing (100 µL working volume). Following resuspension, the cells were stained with APC-conjugated LCMV.GP34p-MHC tetramers at a 1:100 dilution on ice for 30-45 minutes. The cells were then treated with FcR Block (eBioscience, 16-0161) at a 1:100 dilution for 15 minutes on ice, then labeled with FITC-conjugated anti-CD8a (clone 53-6.7, eBioscience, 11-0081) and PE- or PECy7-conjugated anti-CD4 (clone RM5-4, eBioscience, 12-0042 or 25-0042, respectively) at a 1:100 dilution for 30 minutes on ice.

Cells were then washed with 10-fold excess of PBS containing 0.1% BSA and 2 mM EDTA and centrifuged at 300 x g for 5 minutes at 4°C. Cell pellets were resuspended at a 200-300 µL working volume and were analyzed using FACSAria™IIIu (BD Biosciences) and FACSDiva Software (BD Biosciences).

4.3.7 T Cell-Mediated Granzyme B Activity of Enriched LCMV.GP34-Specific $CD8^+$ T Cells Against LCMV.GP34 Peptide-Loaded EL-4 Target Cells

T cell-mediated granzyme B activity was determined using GranToxiLux PLUS! (OncoImmunin). Briefly, target EL-4 (ATCC®TIB-39™) lymphoblast cells were resuspended at a concentration of 2 x 10^6 cells/mL in RPMI 1640 containing 10% FBS (complete RPMI1640). Cells were loaded with LCMV.GP34 peptide (50 µg/mL) for 1 hour at 37°C. Cells were loaded with the cell-permeable fluorescent TFL4 dye (provided by the manufacturer) at a 1:1000 dilution during the last 15 minutes of incubation. The cells were washed with 10-fold excess of complete RPMI 1640, pelleted, and resuspended in complete RPMI 1640. Effector T cells from expansion cultures were counted and resuspended in 100 µL of complete RPMI 1640. Target cell volume was adjusted to yield 100 µL working volumes, and to yield a final effector to target (E:T) ratio of 5:1. Effector cells (in 100 µL) were added to round-bottom 96-well plates, and target cells (in 100 µL) were added immediately to those wells. The plate was centrifuged for 5 minutes at 300 x g and the media was removed by deftly flicking the plate. 75 µL of granzyme B substrate (provided by manufacturer) was added to the pelleted cells. Effectors and targets were co-cultured for 1 hour at 37°C in 5% CO_2. Cells were transferred to 12 x 75 mm test tubes, washed with PBS containing 0.1% BSA and 2 mM EDTA, and centrifuged at 300 x g for 5 minutes at 4°C. Flow cytometric analysis was done on a FACSAriaIIIu (BD Biociences). Voltages were adjusted so that background granzyme B fluorescence (from target cells + granzyme B substrate only) was between

10^3 and 10^4 random fluorescence units (RFUs). Further analysis was performed using FlowJo 7.6.5 (Treestar®, Inc.)

4.4 RESULTS

4.4.1. DP Thymocyte Sorting

For all experiments, DP thymocytes were sorted using FITC-conjugated anti-CD8 and PE-conjugated or PECy7-conjugated anti-CD4 antibodies. DP cells consistently ranged from 70-80% of the live thymocyte population (Figure 4.2).

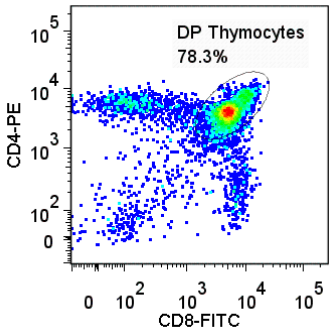

Figure 4.2: Sorted DP thymocytes consistently represented 70-80% of the live thymocyte population. This figure is representative of all DP cell sorts.

4.4.2. $CD8^+$ SP T Cell Generation in the Presence or Absence of Class I Tetramers

After 7 days of differentiation, the percentage of $CD8^+$ SP T cells obtained was consistently over 50% of the total live population, regardless of the presence or type of Class I tetramers (Figure 4.3). In all experiments, negligible amounts of $CD4^+$ SP T cells were found (< 1% of the live cell population). Of the $CD8^+$ SP T cells, generation of an antigen-specific population was variable and appeared to be dependent on the type of tetramer used to induce differentiation.

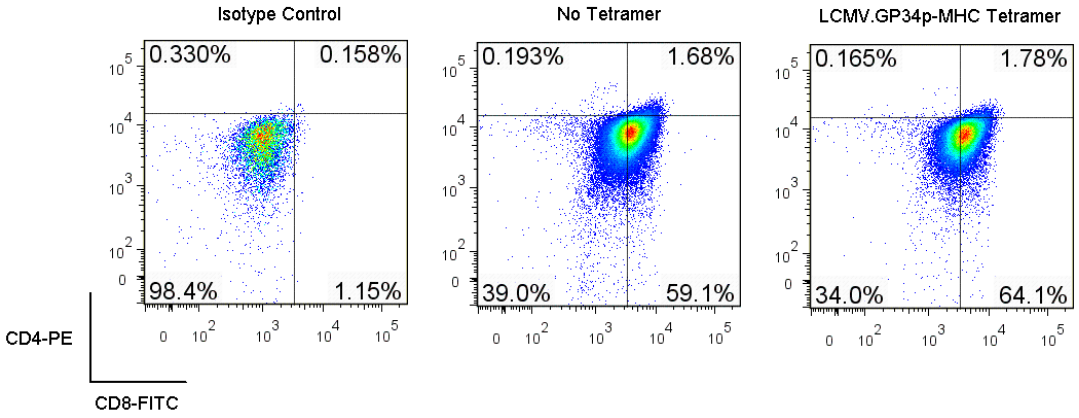

Figure 4.3: $CD8^+$ SP T cells consistently made up over 50% of the differentiated T cell population, regardless of the presence (right) or absence (middle) of tetramers. Minimal $CD4^+$ SP T cells were generated. These plots are representative of all experiments performed.

4.4.3. Generation of OVA257-Specific $CD8^+$ T Cells from DP Thymocytes Using OVA257p-MHC Class I Tetramers

Due to the wide use of OVA as a model antigen and the accessibility of OVA epitope-specific TCR transgenic mice and tumor models, initial experiments to generate OVA257-specific $CD8^+$ SP T cells from C567BL/6 T cell progenitors were attempted. It was hypothesized that the presence of OVA257p-MHC Class I tetramers would induce the generation of OVA257-specific $CD8^+$ T cells that could be further expanded. After 7 days of being "trained" with or without OVA257p-MHC Class I tetramers, the majority of DP thymocytes differentiated into SP $CD8^+$ T cells (70.8% and 91.2%, respectively); however, the percentage of OVA257-specific $CD8^+$ T cells generated using OVA257p-MHC Class I tetramers (2.07%) was slightly lower compared to the DP thymocytes differentiated without tetramers (3.88%) (Figure 4.4).

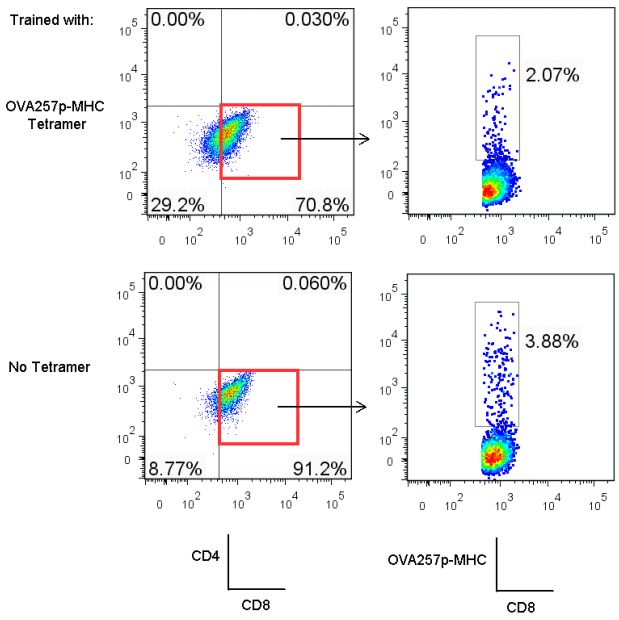

Figure 4.4: CD8$^+$ SP cells (left column) and OVA257-specific CD8$^+$ SP T cells (right column) generated using OVA257p-MHC Class I tetramers (top row) or no tetramers (bottom row).

4.4.4. Differentiation of OVA257- or LCMV.GP34-Specific CD8$^+$ T Cells from DP Thymocytes Using OVA257p-MHC or LCMV.GP34p-MHC Class I Tetramers

This data was discouraging, as it suggested that OVA257p-MHC tetramers were not inducing antigen-specific differentiation, and might even be deleting high-avidity OVA257-specific cells. To determine whether this phenomenon would occur with other tetramers, DP thymocytes were differentiated with OVA257p-MHC or LCMV.GP34p-MHC tetramers (Figure 4.5).

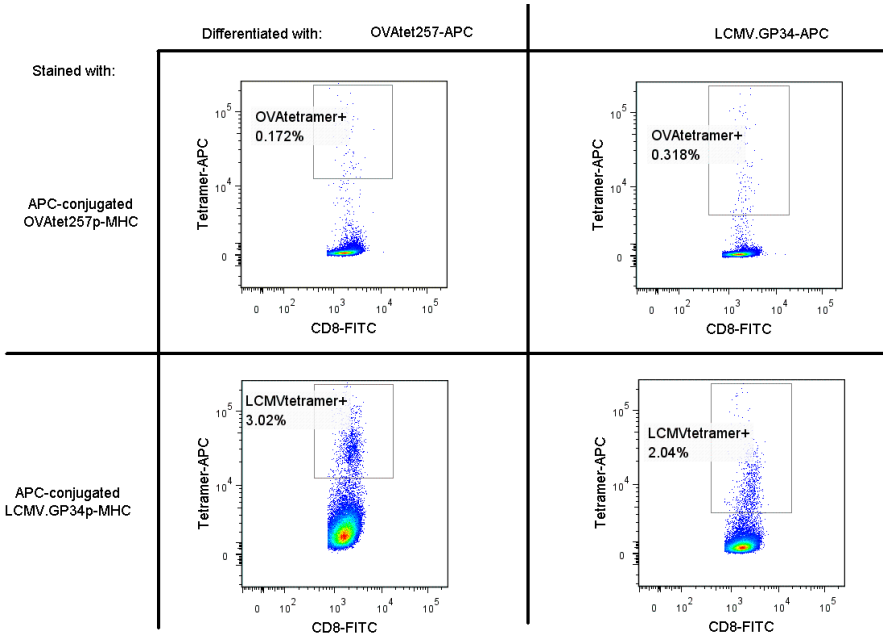

Figure 4.5: OVA257- and LCMV.GP34-specific $CD8^+$ T cells generated from culture with OVA257p-MHC or LCMV.GP34p-MHC Class I tetramers. The antigen-specific populations were sorted and expanded with the respective tetramers.

Unexpectedly, when DP thymocytes were trained with OVA257p-MHC for 7 days, a higher percentage of the $CD8^+$ SP cells were LCMV.GP34-specific (approximately 3.02%, Figure 4.5 lower left) compared to OVA257-specific (approximately 0.172%) (Figure 4.5 upper left). Interestingly, this trend was not seen when LCMV.GP34p-MHC tetramers are used to differentiate the DP thymocytes. After 7 days of culture with the LCMV.GP34 tetramers, a higher percentage of cells were LCMV.GP34-specific (approximately 2.04%, Figure 4.5 lower right) compared to OVA257-specific (approximately 0.318%, Figure 4.5 upper right). Similar results have been observed using OVA257p- and LCMV.GP34p-MHC-functionalized microbeads for differentiation (detailed in Chapter 5).

4.4.5. Expansion of OVA257- or LCMV.GP34-Specific CD8$^+$ T Cells Using OVA257p-MHC or LCMV.GP34p-MHC Class I Tetramers

Despite the puzzling differentiation results, expansion of SP CD8$^+$ T cells was attempted. OVA257- and LCMV.GP34-specific CD8$^+$ SP cells from all groups were sorted and expanded for 7 days with the respective tetramers and co-stimulatory factors (Figure 4.6). Flow cytometry analysis indicates that the majority of the sorted OVA257-specific CD8$^+$ SP T cells that were expanded with OVA257p-MHC Class I tetramers retained OVA257-specificty (88.7% and 78.7%, Figures 4.6A, C), but were also LCMV.GP34-specific (93.8% and 82.9%, Figures 4.6E, G). After expansion of the sorted LCMV.GP34-specific CD8$^+$ T cells with LCMV.GP34p-MHC Class I tetramers, almost all of the CD8$^+$ SP cells were LCMV.GP34-specific (95.3% and 93.2%, Figures 4.6F, H) compared to OVA257-specific (41.4% and 13.6%, Figures 4.6B, D). Due to the cross-reactivity of cells expanded with OVA257p-MHC tetramers, only LCMV.GP34 tetramers were used in further experiments. Cells counts indicated that cell number did not increase within the 7 days of culture.

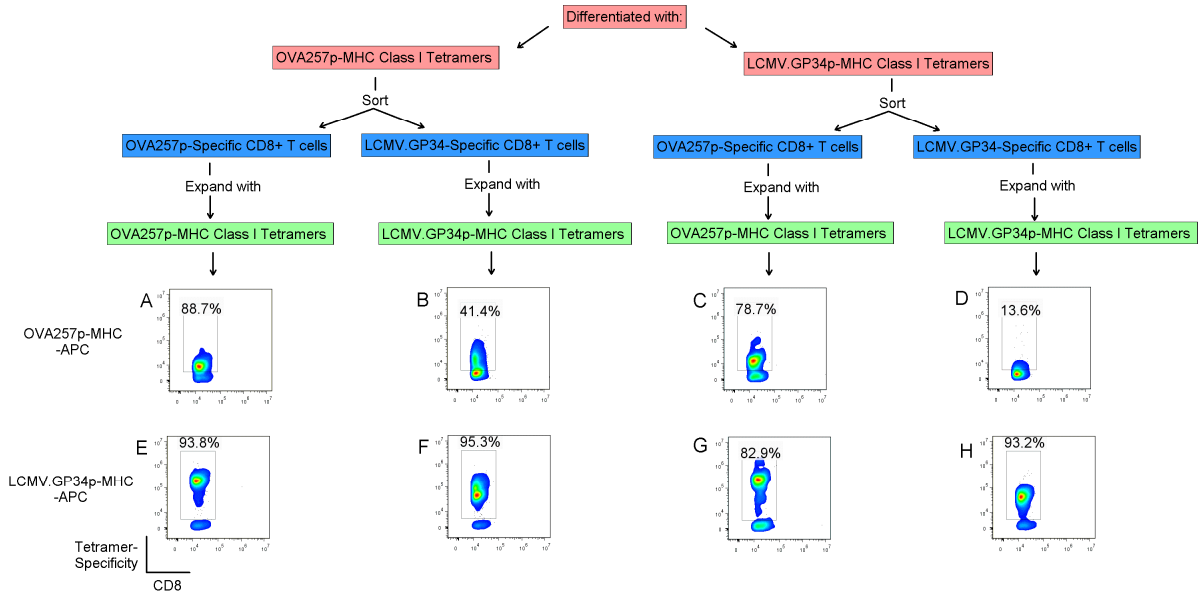

Figure 4.6: OVA257- (A-D) or LCMV.GP34- (E-H) specificity of DP thymocytes after differentiation with OVA257p-MHC (A,B,E,F) or LCMV.GP34p-MHC tetramers (C, D, G, H) followed by expansion with OVA257p-MHC tetramers (A,C,E,G) or LCMV.GP34pMHC tetramers (B, D, F, H).

4.4.6. Generation of LCMV.GP34-Specific CD8$^+$ T Cells Using LCMV.GP34p-MHC Class I Tetramers

In a separate experiment, using only LCMV.GP34p-MHC Class I tetramers to induce differentiation, approximately 46.4% of CD8$^+$ SP T cells were LCMV.GP34-specific after 7 days of differentiation (Figure 4.7). Live LCMV.GP34-specific CD8$^+$ T cells and non-specific CD8$^+$ SP T cells from cultures differentiated with LCMV.GP34p-MHC Class I tetramers were sorted. Dead cells were excluded from sorting by 7AAD staining.

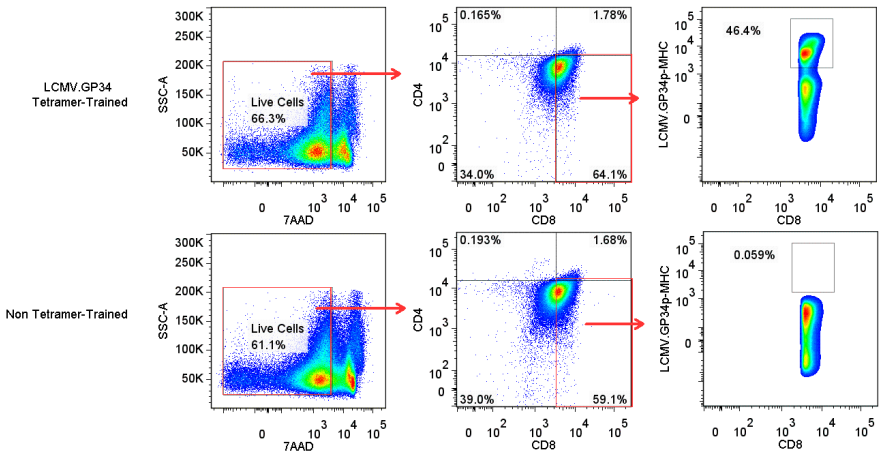

Figure 4.7: CD4 and CD8 expression and LCMV.GP34-specificity of DP thymocytes differentiated with LCMV.GP34p-MHC Class I tetramers (top row) or no tetramers (bottom row). LCMV.GP34-specific and non-specific cells from LCMV.GP34 tetramer-trained cultures were sorted.

4.4.7. Expansion of LCMV.GP34-Specific CD8$^+$ T Cells Using LCMV.GP34p-MHC Class I Tetramers

The sorted LCMV.GP34-specific and non-specific CD8$^+$ T cells were expanded in media with or without LCMV.GP34p-MHC Class I tetramers, respectively, supplemented with anti-CD3, anti-CD28, and IL-2. After expansion, cells were analyzed by flow cytometry to confirm maintenance of tetramer specificity (Figure 4.8). Interestingly, two separate populations appeared in the FSC versus SSC of cells expanded without tetramers. The larger (higher FSC) population contained mostly dead cells, as confirmed by 7AAD$^+$ staining. As expected, the majority of the LCMV.GP34-specific CD8$^+$ SP T expanded with LCMV.GP34p-MHC Class I tetramers retained their antigen-specificity. Non-specific CD8$^+$ SP T cells expanded without tetramers showed minimal levels of LCMV.GP34p-MHC tetramer staining.

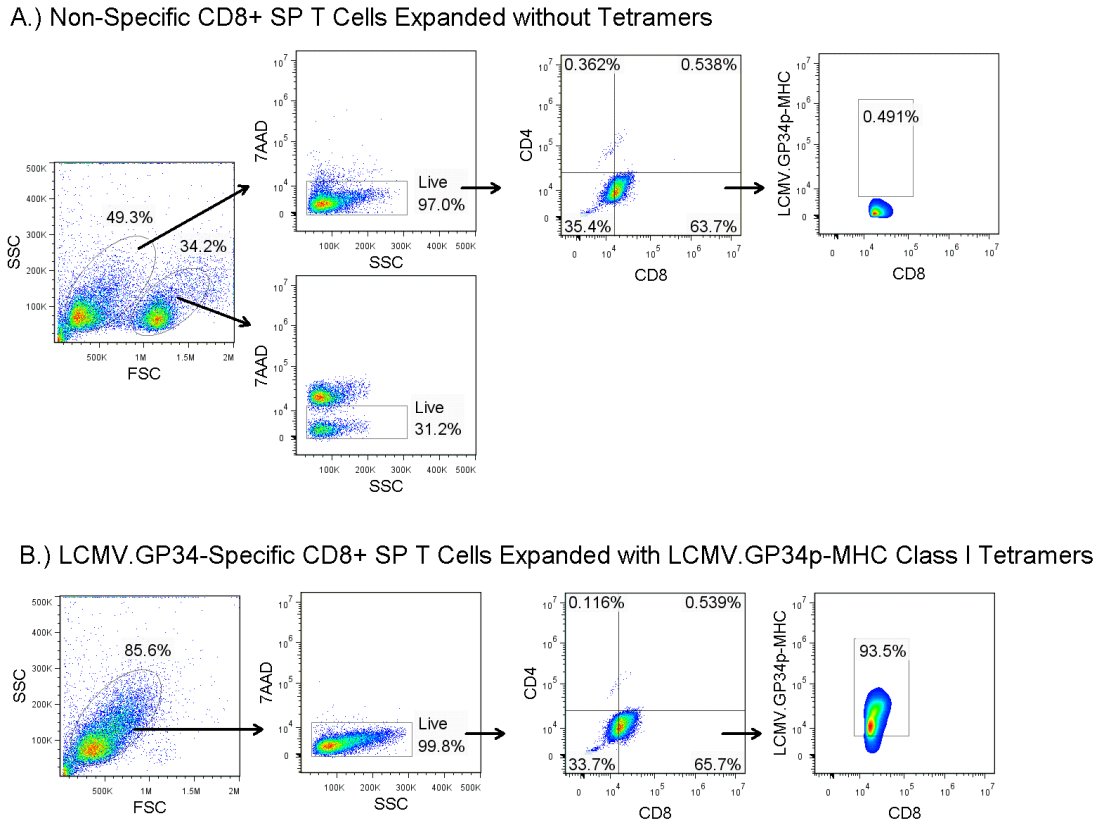

Figure 4.8: A) LCMV.GP34-specificity of non-specific $CD8^+$ SP T cells expanded without tetramers and B) LCMV.GP34-specificity of LCMV.GP34-specific $CD8^+$ T cells expanded with LCMV.GP34p-MHC Class I tetramers.

During all differentiation experiments utilizing LCMV.GP34p-MHC tetramers, 30,000 to 100,000 antigen-specific $CD8^+$ SP T cells were consistently obtained. Despite the enrichment of antigen-specific $CD8^+$ T cells and the use of Class I tetramers, anti-CD28, anti-CD3, and IL-2 to induce expansion, cell numbers remained the same at the end of the 7 day expansion culture. 7AAD staining confirmed that the antigen-specific $CD8^+$ T cells were viable after 7 days of culture in expansion media containing tetramers, anti-CD28, anti-CD3, and IL-2 (Figure 4.8).

4.4.8. Progenitor-Derived LCMV.GP34-Specific CD8$^+$ T Cells Differentiated and Expanded with LCMV.GP34p-MHC Class I Tetramers Remain Functional

To determine if LCMVp.GP34-specific T cells remained functional after 14 days of differentiation and expansion, T cell-mediated granzyme B activity was evaluated. Following the 14 days of culture detailed above, LCMV.GP34-specific or non-specific CD8$^+$ SP T cells were co-cultured with LCMV.GP34 peptide-loaded EL-4 target cells for 1 hour. Prior to co-culture, the EL-4 targets were labeled with a TFL4 dye and loaded with LCMV.GP34 peptide. Addition of cell-permeable granzyme B substrate was added immediately upon co-culture. The substrate fluoresces when cleaved by granzyme B.

Non-specific CD8$^+$ T cells, co-cultured with LCMV peptide-loaded EL-4 target cells, exhibited minimal levels of granzyme B activity (2.39%, Figure 4.9 middle row). However, approximately 28.8% of EL-4 target cells cultured with LCMV.GP34-specific effector T cells contained active granzyme B (Figure 4.9 bottom row), confirming that the LCMV.GP34-specific CD8$^+$ T cells, but not the non-specific CD8$^+$ T cells, can induce the killing of LCMV.GP34 peptide-loaded target cells. This data suggests that although the antigen-specific T cells appear to have lost expansion and/or proliferative capacity, they retained cytotoxic functionality.

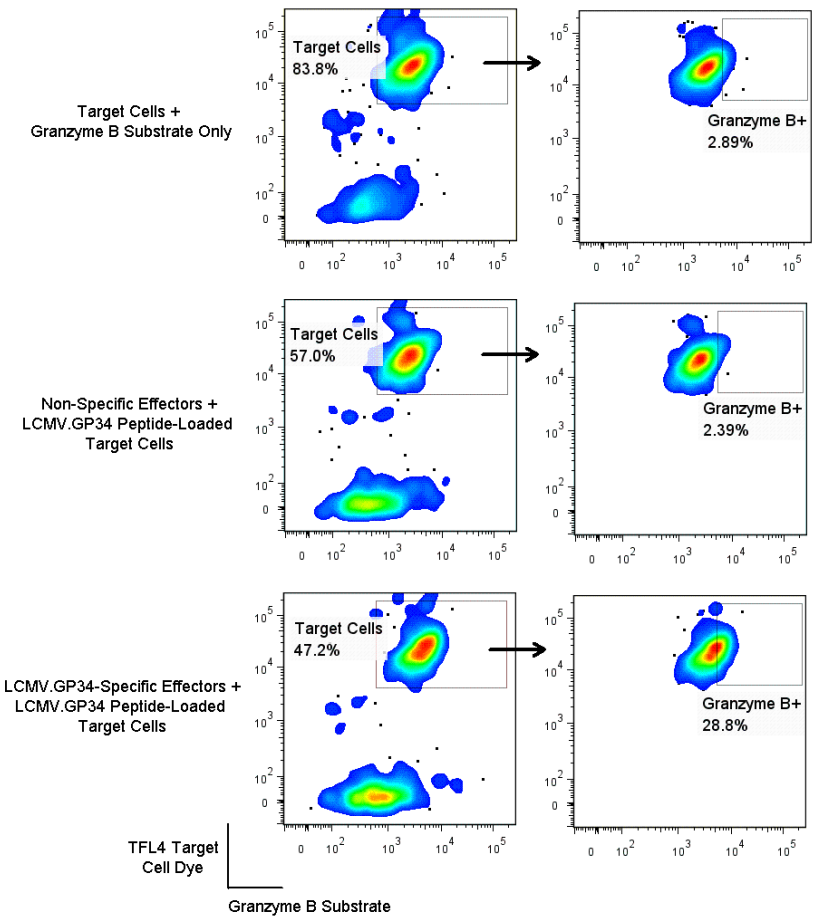

Figure 4.9: T cell-mediated granzyme B activity of non-specific (middle row) and LCMV.GP34-specific (bottom row) $CD8^+$ SP T cells against LCMV.GP34 peptide-loaded EL-4 target cells.

4.5 DISCUSSION

One surprising finding from these studies was the quantitative difference in antigen-specific differentiation induced by OVA257p- and LCMV.GP34p-MHC Class I tetramers. While both tetramers induced $CD8^+$ SP T cell generation with equal efficiency, differentiation with OVA257p-MHC tetramers resulted in a higher percentage of LCMV.GP34-specific $CD8^+$ SP T cells than OVA257-specific $CD8^+$ T cells. However, differentiation with LCMV.GP34p-MHC tetramers resulted in a higher percentage of LCMV.GP34-specific $CD8^+$ SP T cells than OVA257-specific $CD8^+$ T cells. From the results, it was hypothesized that instead of inducing OVA257-specific differentiation, the OVA257p-MHC tetramer, but not the LCMV.GP34p-MHC tetramer, skewed the T cell population to other antigen-specificities. In the following section, reasons for how and why this could happen are presented.

4.5.1 Thymocyte Population Bias for OVA257p-MHC Class I Tetramers

It was hypothesized that C57BL/6 DP thymocytes undergo higher levels of negative selection when cultured with OVA257p-MHC tetramers compared to the LCMV.GP34p-MHC tetramers. This may attributed to the inherent repertoire of C57BL/6 mice if it contains a population of thymocytes with TCRs that bind with high avidity to the OVA257p-MHC tetramer. Indeed, staining of freshly isolated C57BL/6 thymocytes shows that approximately 7% of the thymocytes bind to the OVA257p-MHC tetramer, compared to minimal binding to the LCMV.GP34p-MHC tetramer (< 0.5% of thymocyte population) (Figure 4.10). Further analysis indicates that the majority (97.4%) of the OVA257p-MHC tetramer-binding population are DP thymocytes.

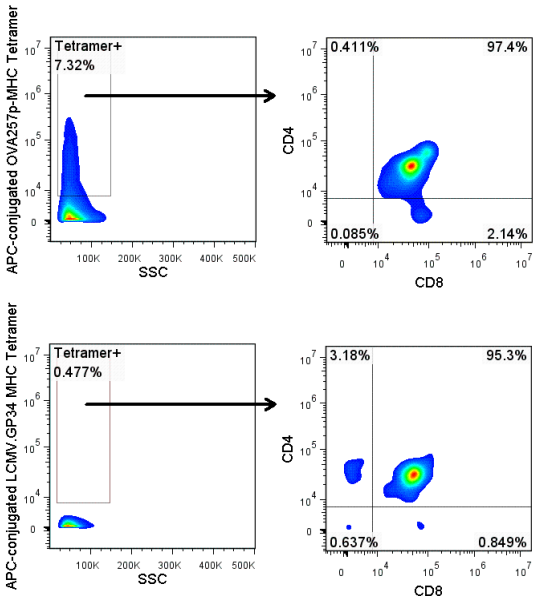

Figure 4.10: OVA257- (upper left) and LCMV.GP34- (lower left) specificity of C57BL/6 thymocytes. The majority of tetramer-binding cells are DP thymocytes (right column).

TCR repertoire analysis of C57BL/6 mice indicates that Vβ1-1, Vβ5-2, Vβ12-1, and Vα2-1 are more highly represented in DP and $CD8^+$ SP T cells over other Vα or β families.[24] Interestingly, naïve $CD8^+$ T cells from Vβ5-2 transgenic mice generate very strong immune responses to OVAp-H-$2K^b$ complexes *in vitro*, and oligoclonal CTLs derived from non-transgenic mice have dramatically skewed expression of Vβ5-1 and Vβ5-2.[25] In addition, Vβ5-2 transgenic mice express 30-fold more OVAp-H-$2K^b$ specific $CD8^+$ T cells compared to their non-transgenic littermates.[25] Indeed, OT-1 transgenic mice, which express TCRs that recognize OVA257p-MHCs, contain TCR-Vα2 and TCR-Vβ5 transgenic inserts. Studies have also shown that OVA257-specific $CD8^+$ memory T cells are found in germ-free, unprimed C57BL/6 mice.[26] Collectively, these data suggest the C57BL/6 mice naturally contain a population of thymocytes with TCRs that bind with high avidity to OVA257p-MHC tetramers.

Results indicate that culture with OVA257p-MHC tetramers yields a distinct population of LCMV.GP34-specific cells. It is suspected that the OVA257p-MHC tetramers enriched the LCMV.GP34-specific cells by deleting the population of thymocytes which bind with high avidity to OVA257p-MHC, and simultaneously providing a positively selecting signal for thymocytes with weak to high avidities for LCMV.GP34-H-2K^b molecules. Interestingly, culture of DP thymocytes with LCMV.GP34p-MHC tetramers yielded more LCMV.GP34-specific T cells compared to OVA257-specific T cells. It was hypothesized that the LCMV.GP34p-MHC tetramers enriched for cells with weak to medium avidities for LCMV.GP34-H-2K^b molecules, while cells with avidities for OVA257p-H-2K^b molecules underwent death-by-neglect.

Further studies need to be done to fully understand why LCMV.GP34p-MHC and OVA257p-MHC tetramers affected the C57BL/6 DP thymocyte population differently. One interesting experiment to determine how tetramer avidity affects antigen-specific differentiation could be done using cohorts of OVA-tetramers bearing peptide variants of the SIINFEKL epitope. These tetramers could be used to differentiate antigen-specific T cells with varying avidities for the SIINFEKL pMHC, which could then be sorted and tested for functionality. If the thymocyte population of C57BL/6 mice is truly biasing differentiation, differentiation with low-avidity tetramers should yield high avidity SIINFEKL pMHC-specific T cells. Additional differentiation experiments using tetramers with diverse epitopes should also be done to confirm if antigen-specific T cells can be generated for multiple epitopes in a tetramer-directed fashion.

It also should be noted that the OVA257-specific T cells obtained from expansion cultures were cross-reactive with LCMV.GP34p-MHC tetramers. This finding is important because it confirms that *in vitro*-differentiated T cells must be screened for unexpected cross- or self-reactivity before transplantation. This could be done by

culturing differentiated/expanded cells with autologous PBMCs and screening for cells that undergo proliferation.

4.5.2 T Cell Dysfunction May Prevent Expansion

Another concern of these experiments is that the antigen-specific cells did not efficiently expand after sorting. Adoptively transferred cells must be expanded to the order of billions (approximately 27 doublings of 1 naïve T cell) before being infused into a recipient.[27] Live/dead staining using 7AAD indicates that the LCMV.GP34-specific $CD8^+$ T cells are still alive after 14 days of culture, and the T cells remained functional, as they mediated granzyme B activity in peptide-loaded EL-4 targets. While further studies are needed to determine why the cells did not expand, it is hypothesized that the antigen-specific T cells entered a state of dysfunction. Possible states of dysfunction are discussed in the following sections.

4.5.2.1 T Cell Anergy

One possibility for the failed expansion of the antigen-specific $CD8^+$ T cells *in vitro* is that they became anergic. Anergy is a state of T cell hyporesponsiveness that results from suboptimal stimulation, such as the engagement of the TCR by antigen without co-stimulation from CD28 or pro-inflammatory cytokines such as IL-2. In the anergic state, T cells activate the expression of genes that produce proteins to block TCR signaling and inhibit cytokine production, in particular IL-2, and proliferation.

Studies done by Redmond et al. indicate that ligand density and TCR signaling strength influence T cell tolerance and anergy.[28-30] In the absence of inflammatory signals, high doses of cognate antigen can induce hyporesponsiveness in TCR transgenic mice, and can prevent clonal deletion during additional antigenic stimulation; however, T cells treated with consecutive low antigen doses are effectively deleted.[28,29] In addition,

Smith and colleagues have shown that high affinity peptides can promote T cell unresponsiveness in TCR transgenic mice in the absence of inflammatory signals, while low affinity peptides induce T cell deletion.[31] However, because anti-CD28 and IL-2 were consistently and continuously used in the expansion media of the system described, lack of co-stimulation is most likely not a problem.

4.5.2.2 T Cell Tolerization

Due to the treatment of DP thymocytes with tetramers, it is possible that high-avidity $CD8^+$ T cells were negatively selected, resulting in a pool of "tolerant" weak-avidity antigen-specific cells. In addition, the SP T cells derived from differentiation cultures may have become tolerized due to chronic stimulation with the tetramers, co-stimulatory molecules anti-CD3, anti-CD28, and IL-2. It is known that tolerized T cells are antigen-experienced and enter a plastic, non-proliferative state. Peripheral tolerance can be induced by continual exposure of T cells to self-antigen, but does not necessarily disrupt effector functions and can be reversed *in vitro* by inducing proliferation with IL-15 or *in vivo* via lymphopenia.[31-35] The observed results, lack of expansion with retained functionality, suggest that antigen-specific T cells may have become tolerized during differentiation or expansion.

Tolerized T cells are characterized by a tolerance-specific gene program that is unique from that of naïve or memory T cells. To determine the genome-wide differences between tolerized and non-tolerized T cells, Schietinger and colleagues "rescued" tolerized T cells by transferring them into lymphopenic mice.[34,36] Genome-wide analysis of the tolerized and "rescued" T cells indicates that tolerant T cells have uniquely overexpressed genes modulating effector function, cell cycle, division, nucleosome assembly, mitosis, and DNA replication. Tolerized T cells have reduced expression of

genes controlling effector molecules interferon gamma (IFNγ), perforin 1 (PRF1), and granzyme M (GZMM), as well as increased gene expression of chemokine and chemokine receptors, CXCR3, CCR5, and CCL5.[36] Compared to rescued T cells, tolerized T cells have lower expression of the master transcription factors, T-bet, EOMES, GATA3, and STAT4, and increased expression of the negative transcriptional regulators, early growth response protein 1 (EGR1) and EGR2.[36] Schietinger et al. also showed that tolerized T cells are re-tolerized in non-tolerogenic hosts, suggesting that the regulation of tolerance-specific genes occurs on an epigenetic level. Indeed, it was found that tolerized and re-tolerized T cells have increased expression of genes regulating chromatin modification including DNA-methyltransferase 1 (DNMT1), histone acetyl transfer 1 (HAT1), histone deacetylase 2 (HDAC2), and HDAC3.[36]

4.5.2.3 T Cell Exhaustion

T cell exhaustion arises from chronic stimulation caused by prolonged infections or cancer and represents a terminal state of T cell differentiation characterized by hyporesponsiveness. Exhaustion can be induced by chronic antigenic stimulation; however, unlike anergy, which happens rapidly after stimulation, T cell exhaustion is a progressive process characterized by hierarchical stages.[30,34,37-39] $CD8^+$ T cells first lose their proliferative capacity and ability to produce IL-2. Severely exhausted $CD8^+$ cells fail to produce IFNγ and are unable to degranulate.[30,34,39] Distinct from memory T cells, which can persist without antigen, via IL-7 and IL-15-mediated homeostasis, exhausted T cells have low expression of CD122 (the IL-15 receptor β-chain) and CD127 (the IL-7 receptor α-chain).[30,34,39] They require tonic signaling from the cognate antigen to survive, a term coined "antigen addiction".[30,34,39] Exhausted T cells are characterized by loss of

effector function, sustained expression of inhibitory receptors, and a distinct transcriptional state.[30,34,39-41]

PD-1 has been implicated as the main inhibitory receptor regulating T cell exhaustion. Persistent antigen stimulation causes epigenetic alterations in the *Pdcd1* locus, resulting in the long-term expression of PD-1 on antigen-specific T cells.[42] The PD-1/PDL-1 signaling pathway has been shown to attenuate functional and proliferative capacities of T cells by repressing TCR signaling and inducing the expression of genes that impair T cell function.[42] PD-1 signaling has also been shown to inhibit T cell motility, thus preventing $CD8^+$ T cells from exhibiting proper effector function and target cell killing.[42] Exhausted $CD8^+$ T cells represent a heterogenous population of cells, T-$bet^{hi}PD\text{-}1^{int}$ and $EOMES^{hi}PD\text{-}1^{hi}$ cells.[39] T-$bet^{hi}PD\text{-}1^{int}$ $CD8^+$ T cells are a progenitor subset that proliferate in response to persisting antigen and give rise to $EOMES^{hi}PD\text{-}1^{hi}$ $CD8^+$ T cells, the terminally-differentiated progeny. $EOMES^{hi}PD\text{-}1^{hi}$ $CD8^+$ T cells have higher inhibitory receptors and do not replicate but express high levels of cytotoxic activity.[39] In addition to PD-1 signaling, myeloid-derived suppressor cells (MDSCs) as well as $FoxP3^+CD4^+$ regulatory T (T_{REG}) cells, which secrete immunosuppressive cytokines such as IL-10 and TGF-β, induce and maintain T cell exhaustion.[39]

In addition to PD-1 overexpression, exhausted T cells co-express inhibitory receptors LAG-3, CD244, CD160, TIM-3, and CTLA-4, and have low expression of CD122 (the IL-2 and IL-15 β-chain receptor) and CD127 (the IL-7 α-chain receptor).[34,39] Genomic approaches have provided much insight into the molecular program of exhausted $CD8^+$ T cells and have confirmed that they are transcriptionally distinct from naïve or memory T cells.[39] The transcriptional repressor, Blimp-1, has been implicated in T cell exhaustion.[43,44] In small amounts, Blimp-1 is found to promote the formation of memory T cells, and intermediate levels of Blimp-1 can induce terminal differentiation of

functional effectors.[45] Blimp-1 is found in very high levels in exhausted T cells, and is associated with the upregulation of the previously described inhibitory receptors. Exhausted T cells also express higher levels of NFATc1, which has been shown to regulate PD-1 expression *in vitro*.[40,43,44]

4.5.2.4 T Cell Senescence

Lastly, it is possible that the T cells failed to expand because they were senescent. Senescent T cells are characterized by telomeric shortening, which ultimately results in permanent cell cycle arrest.[30,46-49] While T cell senescence is naturally associated with aging, high levels of senescent T cells are found in young patients with autoimmune diseases, suggesting that chronic activation and proliferation may induce T cell senescence. Senescent T cells are characterized by the loss of the important co-stimulatory receptor, CD28, which is essential for T cells functions such as lipid raft formation, IL-2 gene transcription, cell adhesion, and glucose metabolism.[30,46-49] Interestingly, although senescent $CD8^+$ T cells cannot replicate in response to TCR stimulation, either by the cognate antigen or mitogens, they contain high levels of perforins and granzymes and retain some functional capacity, including IFNγ production and cytoxocity.[30,46-49]

Senescent T cells are characterized by the loss of CD28 and re-expression of CD45RA. $CD8^+$ T cells that are senescent have a characteristic phenotype ($CD45RA^+CD28^-CD25^-CD69^-CCR7^-CD62L^-CD57^+KLRG1^+$).[46] Diminished expression of CD28 results in decreased TCR signaling and reduced ability to secrete IL-2. In addition, CD40L expression is significant lower in $CD28^-CD8^+$ T cells. Interestingly, $CD28^-CD8^+$ T cells demonstrate enhanced expression of co-stimulatory molecules 4-1BB, CD244, and SLAMF7.[46] It is thought that the simultaneous loss and enhancement

of co-stimulatory receptors serves as a compensatory mechanism, but this phenomenon is not completely understood. Real-time polymerase chain reaction (RT-PCR) analysis and phenotyping has shown that $CD28^-CD8^+$ T cells also express higher levels of NK cell stimulatory receptors such as CD16, CD56, KIR2DL2, CD94, NKG2D, and KLRG1 compared to their $CD28^+CD8^+$ memory T cell counterparts.[50] Cytokines, chemokines, and chemokine receptors are also expressed differentially in $CD28^-CD8^+$ and $CD28^+$ $CD8^+$ T cells. Gene expression analysis has shown that IL-3, IL-23A, IL-7R, and IL-12RB2 mRNA levels are higher in $CD28^+CD8^+$ T cells, while IL-12A and IL-13 mRNA levels are higher in $CD28^-CD8^+$ T cells, suggesting different proliferative requirements for both sets of memory T cells.[50] CCR2, CCR6, CCR7 mRNA levels are higher in $CD28^+CD8^+$ T cells, while CCL4 and CXCR1 levels are higher in $CD28^-CD8^+$ T cells.[50]

Senescent T cells are characterized by shortened telomeres.[46,50] Telomere shortening is an inevitable process that occurs with cell replication. When the telomere shortens to its critical length, DNA damage signals result in cell cycle arrest and replicative senescence.[46,50] This mechanism is thought to protect cells from malignant changes brought about by the chromosomal instability of dividing cells with shortened telomeres.[46,50] The loss of CD28 parallels telomere shortening in senescent T cells.[46,50] Telomeric sequences can be restored by the action of telomerase. Lymphocytes have the ability to upregulate telomerase in response to proliferative stimuli such as acute viral infections or when activated by anti-CD3 or anti-CD28 antibodies; however, after repeated rounds of stimulation, telomerase activity in $CD8^+$ T cells declines and becomes undetectable.[46,50-52]

4.5.2.5 Preventing T Cell Dysfunction

Progenitor-derived antigen-specific $CD8^+$ T cells should be evaluated, both molecularly and cellularly, to determine the most appropriate way to prevent dysregulation. As described in the previous sections, tolerized, exhausted, and senescent T cells are characterized by unique phenotypic markers and transcriptional profiles. Before expansion, antigen-specific T cells should be screened by flow cytometry for various dysfunction markers. In addition, quantitative RT-PCR would provide a deeper understanding of the molecular state of the cells.

The failure to increase cell number may be due to lack of proliferation; however, it is also possible that some cells are proliferating while similar numbers are dying, resulting in unchanged cell numbers after culture. To discriminate between these two possibilities, a proliferation assay could be done. Cells could be labeled with CFSE and monitored by flow cytometry over the course of 7 days to determine if the cells have proliferative capacity.

As discussed in the previous sections, various forms of T cell dysregulation can be caused by chronic stimulation, resulting in the loss of proliferative capacity. The amounts of tetramers, co-stimulatory molecules, and cytokines used for differentiation and/or expansion might provide too much stimulation and hinder proliferation.[31,32,37,38] One solution is to adjust the concentrations of the tetramers, anti-CD3, anti-CD28, and IL-2 to provide optimal amounts that allow for antigen-specific $CD8^+$ T cell expansion. Because the antigen-specific $CD8^+$ SP T cells are sorted prior to expansion, it is not necessary to include Class I tetramers during expansion. Elimination of the antigen would reduce chronic TCR signaling and increase the proliferative capacity of the T cells. In addition, lowering the concentration of anti-CD3, anti-CD28, and/or IL-2 would lower

the level of tonic stimulation and might prevent any tolerization that is occurring during the expansion portion of the procedure.

In addition to adjusting the amount of IL-2 in the expansion media, additional cytokines, in particular, IL-15, may need to be incorporated for the T cells to retain proliferative activity.[53-61] Studies have shown that IL-15 induces proliferation of memory $CD8^+$ T cells similar to TCR crosslinking, and is capable of inducing cytotoxic activity in memory CTLs.[55] Gene expression profiling shows similarities between cells stimulated with IL-15 and antigen-mediated activation of memory $CD8^+$ T cells.[60] Teague and colleagues have shown that IL-15 can rescue tolerant $CD8^+$ T cells *in vitro*, and that these rescued T cells can be used effectively for adoptive transfer after stimulation.[58]

PD-1 blockade has been very well-studied and is an effective way to reverse T cell exhaustion of PD-1^{int} $CD8^+$ T cells.[42,62,63] If the antigen-specific $CD8^+$ T cells from the differentiation or expansion cultures express intermediate to high levels of PD-1, *in vitro* culture with antagonist antibodies might prevent exhaustion and help maintain proliferative capacity; however, since the described system only contains T cells, PD-1/PDL-1 signaling is not expected to occur. Additional methods such as blocking other inhibitory receptors such as Tim-3, LAG-3, and CTLA-4 might enhance the expansion capacity of *in vitro*-exhausted T cells.[64]

The epigenetic changes associated with exhaustion or tolerance may be reversible. Studies indicate that $CD8^+$ T cell exhaustion during chronic LCMV infection is associated with a downregulation of diacetylated histone H3 (diAcH3).[65] Zhang and colleagues have found that *in vitro* treatment of exhausted T cells with valproic acid (VPA), an HDAC inhibitor, increases the level of diAcH3 at the *Ifng* and *Il2* promoters by seven- and two-fold, respectively.[65] VPA-treated T cells also produced 50-fold higher amounts of IFNγ and four-fold higher amounts of tumor necrosis factor alpha (TNFα)

mRNA compared to non-treated exhausted T cells.[65] After adoptive transfer, VPA-treated $CD8^+$ T cells survived and differentiated into functional memory T cells that were capable of mounting a response against LCMV challenge.[65] These studies suggest that exhaustion may be prevented or reversed with the use of HDAC inhibitors.

Preventing replicative senescence using gene transduction of human telomerase reverse transcriptase (hTERT), an important component of telomerase, has been well-studied in human fibroblasts, epithelial cells, and keratinocytes.[66-69] Cells transduced with hTERT exhibit telomere stabilization and unlimited proliferation with no evidence of abnormal growth or tumorogenicity in severe combined immunodeficiency (SCID) mice.[66-69] Expressing exogenous hTERT has been attempted with HIV-specific $CD8^+$ T cells using a number of viral vectors; however, these approaches fail to prevent the loss of CD28, indicating that expression of telomerase in combination with CD28 may be required.[70] Potential non-genetic approaches to modulate T cell replicative senescence center on the use of estrogen. Studies show that an estrogen-responsive element is found in the promoter region of IFNγ, a cytokine that has been shown to upregulate telomerase in T cells.[48,71-73] The promoter region of hTERT contains an estrogen-responsive element in various reproductive tissues, and estrogen has been shown to modulate calcium mobilization in T cells.[71] Collectively, these data suggest the potential use of estrogen for preventing T cell senescence.

4.6 CONCLUSION

In this chapter, a method to enrich and expand progenitor-derived $CD8^+$ SP T cells was presented. The studies detailed here indicate that progenitor-derived LCMV.GP34-specific $CD8^+$ T cells can be enriched and cultured for 7 days in expansion media containing tetramers, co-stimulatory molecules, and cytokines. Despite the lack of T cell expansion, enriched antigen-specific T cells retained their cytotoxic functionality. Optimization of differentiation and expansion conditions, by lowering the amounts of tetramers, co-stimulatory molecules, and cytokines used for culture, or by including IL-15 in the expansion media may be needed. Further studies should also be done to confirm that T cells lack proliferative capacity, and whether they enter a state of dysfunction after differentiation or during the process of expansion. Molecular and cellular characterization will provide insight on how to reverse the dysfunction and improve expansion capacity.

4.7 ABBREVIATIONS

HSC – hematopoietic stem cell

CTL – cytotoxic T lymphocyte

APC – antigen-presenting cell

DC – dendritic cell

TCR – T cell receptor

pMHC – peptide major histocompatibility complex

pHLA – peptide human leukocyte antigen

T_H – T helper

CTLA-4 – cytotoxic T lymphocyte associated protein 4

IL - interleukin

aAPC – artificial antigen-presenting cell

TAP1/2 – transporter associated antigen presentation 1/2

ICAM-1 – intracellular adhesion molecule 1

LFA – leukocyte function antigen

PBMC – peripheral blood mononuclear cells

MART-1 – melanoma antigen recognized by T cells-1

gp – glycoprotein

CMV – cytomegalovirus

K32/4-1BB/CD3/28 – K562 erythomyeloid cell line expressing Fcγ receptor, human 4-1BB, coated with anti-CD3 and anti-CD28 antibodies

MP – matrix protein

REP – Rapid Expansion Protocol

SP – single positive

DP – double positive

OVA257p-MHC – ovalbumin epitope (SIINFEKL) pMHC

LCMV.GP34p-MHC – lymphocytic choriomeningitis virus glycoprotein epitope (AVYNFATC) pMHC

PBS – phosphate buffered saline

BSA – bovine serum albumin

EDTA – ethylenediaminetetraacetic acid

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

PE – phycoerythrin

PECy7 – phycoerythrin cyanine 7

APC-labeled – allophycocyanin-labeled

APC-conjugated – allophycocyanin-conjugated

RFU – random fluorescence units

MDSC –myeloid-derived suppressor cell

T_{REG} – regulatory T cell

RT-PCR – real-time polymerase chain reaction

IFNγ – interferon gamma

PRF1 – perforin 1

Gzmm – granzyme M

EGR – early growth response factor

DNMT1 – DNA methyl transferase 1

HAT – histone acetyl transferase

HDAC – histone deacetylase inhibitor

diAcH3 – diacetylated histone H3

VPA – valproic acid

TNFα – tumor necrosis factor alpha

hTERT – human telomerase reverse transcriptase

SCID – severe combined immunodeficiency

SIINFEKL – (serine – isoleucine – isoleucine – asparagine – phenylalanine – glutamic acid – lysine – leucine)

AVYNFATC – (alanine – valine – tyrosine – asparagine – phenylalanine – alanine – threonine – cysteine)

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Chapter 5: The Effects of MHC Density and Presentation on Immature Thymocyte Signaling and Differentiation

5.1 INTRODUCTION

In the periphery, engagement of the T cell receptor (TCR) with cognate peptide major histocompatibility complex (pMHC)/human leukocyte antigen (pHLA) Class I or Class II molecules is essential for the priming, activation, and effector function of $CD8^+$ and $CD4^+$ T cells, respectively. TCR-pMHC engagement is critical for thymocyte development *in vivo*, as it directs of the production of a self-restricted and self-tolerant T cells. In the previous chapters, a novel system to differentiate human $CD34^+$ cord blood hematopoietic stem cells (HSCs) into antigen-specific $CD8^+$ T cells was detailed, and methods to expand progenitor-derived antigen-specific $CD8^+$ T cells were explored. While considerable success to induce antigen-specific $CD8^+$ T cell differentiation was demonstrated in the previous chapters, those systems employed soluble tetramers which do not accurately mimic the cell-based presentation of pMHCs *in vivo*.

In the thymus and periphery, TCR-pMHC engagement occurs via pMHCs displayed on the surface of antigen-presenting cells (APCs). The orientation and density of APC-presented pMHCs is crucial for inducing T cell polarization, adhesion, TCR clustering, and lipid raft formation, all of which influence TCR signaling, T cell differentiation, and effector function. Many studies focus on the effects of pMHC surface presentation and density in the context of mature T cell activation and effector functions. In this chapter, the effects of plate- and bead-immobilized pMHC ligand density and solid-phase presentation on thymocyte signaling and differentiation are evaluated. The following section provides a review of current literature detailing the effects of TCR signaling on thymocyte fate and T cell function, factors governing TCR signaling

strength, and the various methods of acellular pMHC presentation that have been developed to date.

5.1.1 Thymocyte Selection and CD4 Versus CD8 Lineage Commitment

Approximately 98% of double positive (DP) cells will undergo apoptosis during a series of developmental checkpoints known as positive and negative selection which occur in the thymic cortex and medulla, respectively.[1] During positive selection, DP thymocytes undergo "death by neglect" if their TCRs fail to engage pMHCs presented by cortical thymic epithelial cells (cTECs).[1] DP cells that bind to self-antigen presenting Class I or Class II MHCs with moderate avidities mature into $CD8^+$ or $CD4^+$ single positive (SP) T cells, respectively. SP thymocytes migrate to the medullary compartment, where they are eliminated by negative selection if they express TCRs that bind with high avidity to self-antigens.[1] Thymocytes that pass both checkpoints become MHC-restricted and self-tolerant mature SP $CD4^+$ or $CD8^+$ T cells.[1]

5.1.1.1 Anatomical Differences of Positive and Negative Selection

Positive and negative selection is temporally and spatially segregated within the cortical and medullary compartments of the thymus.[2] The cortical and medullary thymic niches contain different types of APCs that use unique, cell-type specific antigen sampling and processing. Cortical TECs use a unique proteasome, β5t, to generate MHC Class I epitopes that are essential for positive selection.[3-5] β5t deficiency does not alter negative selection, suggesting that β5t facilitates the presentation of unique self-epitopes to DP thymocytes within the cortex.[4,5] In an analogous fashion, cTECs utilize cathepsin L and thymus-specific serine protease (TSSP) to display MHC Class II epitopes which are required for positive selection of $CD4^+$ T cells.[6]

The medullary compartment of the thymus is crucial for producing self-tolerant T cells. Much of the medulla's tolerogenic role is attributed to the expression of AIRE, a transcriptional regulator that promotes the promiscuous gene expression of tissue-restricted antigens (TRAs), by medullary thymic epithelial cells (mTECs).[7,8] The medulla also contains various DC subsets that facilitate negative selection and tolerance. In particular, resident conventional DCs (cDCs), which preferentially seed the medulla, display antigens captured within the thymic microenvironment, while migratory cDCs present blood-borne self-antigens.[7]

The repertoire of mature, naïve, SP T cells generated after positive and negative selection is diverse, MHC-restricted, and self-tolerant. Mature T cells exit the thymus and upon encountering foreign antigen in the periphery, undergo dramatic clonal expansion and acquire effector or memory functions. $CD8^+$ SP T cells, or cytotoxic T cells, recognize MHC Class I molecules and are capable of "killing" pathogen-infected cells or tumor cells. $CD4^+$ SP T cells, or T helper (T_H) cells, mediate B cell responses and facilitate cytotoxic T cell activation.

5.1.1.2 The Influence of Signal Strength on Positive and Negative Selection

Thymocyte survival and lineage commitment is highly dependent on the interaction between the developing cell's TCR and pMHC molecules presented by thymic epithelia. These interactions span a wide range of affinities due to the diversity of TCRs and self-peptides displayed by each MHC. Much progress has been made to understand how the strength of TCR signaling determines qualitatively different cell fates.[9-13] In the classic model of thymocyte selection, weak avidity interactions prevent death by neglect, while strong signals result in clonal deletion by negative selection.[13-15]

Experiments using TCR transgenic mice have shown that strong affinity agonist pMHCs induce negative selection, while low affinity antagonists or weak agonists act as positively-selecting ligands. A narrow affinity window for positive versus negative selection has been determined and slight alterations in affinity have been shown to influence the fate of immature thymocytes.[16] The recent development of Nur77-GFP knock-in reporter mice has facilitated the analysis of signal strength during selection on a cell-to-cell basis. Nur77 is an immediate response gene of TCR engagement and is directly proportional to signal strength due to activating stimuli. Work by Moran and colleagues have shown that Nur77 is more highly expressed in cells undergoing negative selection compared to cells undergoing positive selection.[2,17] The Nur77-GFP model has also been used to show that $CD4^+FoxP3^+$ T regulatory (T_{REG}) cells perceive stronger TCR signals than conventional $CD4^+$ T_H cells during development and selection.[17]

Differences in signal strength perceived via the TCR during positive and negative selection are thought to induce differential intracellular kinase pathways that direct thymocyte fate. For example, during positive selection, weak TCR signals result in low, sustained Erk activation, while strong signals that induce negative selection result in transient Erk activation accompanied by JNK and p38 activation.[18-20] The localization of Erk during positive and negative selection also occurs in different intracellular compartments. During positive selection, Erk remains in the Golgi membrane, while it resides in the plasma membrane during negative selection.[21] Knock-down studies have demonstrated differential roles of various kinase and adapter proteins on selection. Deletion of Erk1 and Erk2 results in positive selection defects, but has no effect on negative selection.[22,23] Additionally, knock-down of Grb2, an adaptor protein that mediates kinase activity downstream of TCR engagement, disrupts positive and negative selection, while deletion of Themis, a Grb-2 associated protein, only causes defects in

positive selection.[24] While much is still not fully understood about the role of signal transduction during selection, experiments performed to date highlight the quantitative and qualitative influence of signaling strength on thymocyte fate.

5.1.1.3 The Influence of Signal Strength on the CD4 Versus CD8 Lineage Decision

DP thymocytes expressing both CD4 and CD8 co-receptors are bipotential precursors that differentiate into either $CD4^+$ T_H cells or $CD8^+$ cytotoxic T lymphocytes (CTLs). The decision of lineage fate has been studied for decades, and three "classical" models have been proposed to explain the CD4 versus CD8 lineage choice. One classical model of T lineage commitment, the stochastic selection model, postulates that termination of co-receptor gene expression is a random event during positive selection. According to this theory, only thymocytes with matching TCRs and co-receptors survive positive selection and proceed with differentiation.[25-28]

The instructive strength-of-signal model proposes that the fate of DP thymocytes is determined by the differences in the strength of signaling transduced by engagement of the TCR and co-receptor.[29-31] In this model, quantitative differences in the signals transduced via TCR and co-receptor engagements "instruct" $CD4^+$ or $CD8^+$ lineage commitment. Because CD4 binds to more intracellular Lck than CD8, it is postulated that TCR/CD4 co-engagement results in stronger signals than TCR/CD8 engagement.[31-33] The differences in signal strength result in termination of either CD4 or CD8 expression. The duration-of-signal instructional model is a modified version of the strength-of-signal model, and considers TCR signal duration in the CD4 versus CD8 lineage choice.[34] In this model, the downregulation of CD8 in the intermediate single positive (ISP $CD4^+CD8^{low}$) state disrupts MHC Class I signaling, but not Class II signaling. Therefore, TCR signaling triggered by Class I molecules is shorter, leading to the $CD8^+$ lineage,

while Class II molecules trigger more sustained signaling resulting in $CD4^+$ lineage commitment.[30,34] Both the stochastic and instruction models have been controversial, as experimental observations have been shown to contradict each theory.[35,36]

To date, the best model to explain CD4 versus CD8 lineage commitment is the kinetic signaling model. This model postulates that the CD4 versus CD8 decision is determined by TCR signal duration and that cytokine receptors serve as sensors to detect the duration of TCR signaling.[37-39] In this model, DP thymocytes undergo positive selection and subsequently terminate CD8 gene expression to become $CD4^+CD8^{low}$ ISPs. A decrease in surface CD8 expression abrogates CD8-dependent signaling from MHC Class I molecules. For DP cells to become $CD8^+$ T cells, they must undergo IL-7 dependent co-receptor reversal, whereby CD4 gene expression is terminated and CD8 transcription is reinstated.[37] Studies suggest that cytokine receptor signal transduction functions as a sensor for TCR signaling duration.[30,39-42] Sustained TCR signaling by Class II molecules impairs IL-7R signaling, driving differentiation of ISPs into the CD4 lineage.[37,39,42] If TCR signaling through MHC Class I molecules is disrupted by CD8 downregulation, IL-7R signals initiate co-receptor reversal and $CD8^+$ lineage differentiation.[37,39,42]

5.1.2 T Cell Receptor Engagement and Signaling

The TCR is a heterodimer, composed of an α- and β- chain, that responds to short peptide sequences displayed in the context of Class I and Class II molecules.[43,44] Each TCR chain contains a variable and constant immunoglobulin (Ig)-like domain, transmembrane segment, and short cytoplasmic tail.[43-45] During development, the variable domains are generated via random, somatic recombination of the variable (V), diversity (D), and joining (J) gene segments (D not present on α-chain).[43,44] This

recombination results in the development of T cells with a wide range of specificities and the ability to recognize a diverse range of pMHCs. The αβTCR has no intrinsic signaling capacity, but is associated with three CD3 signaling dimers (δε, γε, and ζζ) that contain immunoreceptor tyrosine-based activation motifs (ITAMs).[46,47] Upon TCR-pMHC engagement, CD3 ITAMs are phosphorylated and recruit other molecules to initiate the downstream signaling cascade which directs T cell development and function.[43,44,46,47]

The CD4 and CD8 co-receptors are indirectly associated with the TCR-CD3ε complex and facilitate the antigen recognition process by binding to the invariant portions of the pMHC during TCR-pMHC engagement.[48,49] The intracellular portions of CD4 and CD8 interact with Lck, thus facilitating signal transduction during early TCR triggering, and are therefore essential for normal T cell activation and function.[32,33,50] Experiments have shown that the absence or blockade of CD4 or CD8 co-receptors decreases TCR sensitivity by more than 10 to 100-fold. In addition to CD4/CD8 co-receptors, co-stimulatory signals provided by CD28 ligation enhance TCR sensitivity to antigen receptor engagement.[51-53] CD28 co-stimulation can increase IL-2 production by 50-fold, and as a result, promotes proliferation in suboptimal mitogenic conditions.[51,52] The extracellular domain of CD28 binds to B7 proteins on APCs, and studies indicate that CD28/B7 ligation induces phosphorylation of tyrosine, serine, and threonine residues of the intracellular portion of CD28, resulting in a subsequent cascade of downstream signaling.[52]

The dynamic nature of the cytoskeleton is an essential component of TCR signaling and activation, as it provides T cells with the ability to migrate and remodel in response to APC contact. Intracellular cytoskeletal changes allow organelle redistribution and polarization during TCR-pMHC engagement and immunological synapse (IS) formation.[54-58] The accumulation of lipid rafts at the IS plays a crucial role in TCR

signaling and T cell activation. These microdomains, formed mainly by sphingomyelin and glycosphingolipids, amplify downstream TCR signaling by facilitating the clustering of TCRs, co-receptors, co-stimulatory molecules, and their associated intracellular signal transducers.[56]

5.1.3 MHC Class I and MHC Class II Molecules

Though MHC Class I and Class II molecules exhibit some structural similarities, they differ in the types of peptides they present and their distributions among cell types.[43,59] Class I molecules are present on all nucleated cells, although their degree of expression varies between cell types and is highest on hematopoietic cells. The peptides that bind to Class I clefts are usually 8-10 amino acids in length and lie elongated within the peptide-binding groove.[43,59] Class I molecules present natural self-antigens or epitopes from pathogens such as viruses or intracellular bacteria, and are recognized by $CD8^+$ CTLs which secrete cytotoxic molecules such as perforins and granzymes to eliminate infected or cancerous cells.[43,59]

MHC Class II molecules present peptides derived from endocytic or exogenous proteins and are normally found on hematopoietic cells, such as B lymphocytes, dendritic cells (DCs), and macrophages, that activate effector $CD4^+$ T_H or T_{REG} cells ,which secrete cytokines to modulate the immune response.[43,59] The peptide-binding groove of Class II molecules is open at both ends and can accommodate longer epitopes, ranging from 11 to 30 amino acids in length.[43,59]

5.1.4 Factors Influencing Cumulative TCR Signaling Strength

During TCR-pMHC engagement, signals transmitted from the extracellular portion of the TCR result in intracellular changes that influence the fate and/or function of T lymphocytes. The binding of TCR to pMHC is highly specific and sensitive, and

subtle quantitative differences in TCR signaling strength can induce different levels of intracellular phosphorylation, resulting in diverse downstream signaling events. The advent of TCR cloning and the development of mice expressing TCRs of known specificity have enhanced the understanding of antigen avidity on TCR signaling. Additionally, the development of multimeric pMHC complexes has provided ligands for biochemical binding/kinetic assays, as well as facilitated the detection of rare, antigen-specific populations.[60] It is known that the affinity, duration, and density of TCR-pMHC interactions contribute to the cumulative strength of TCR signaling *in vivo*.

5.1.4.1 Potency of TCR-pMHC Interactions

In the context of TCR-pMHC engagement, potency refers to the ability of a pMHC ligand to stimulate effector functions such as cytokine production, proliferation, and cytotoxic activity. High potency ligands stimulate numerous effector functions, whereas low potency ligands only stimulate a subset of functions. The potency of TCR-pMHC engagement is dependent on the affinity and half-life ($k_{1/2}$) of the interaction.[61] The relationship between TCR affinity and potency was first noted by Syuklev et al. when they found that the affinities of six pMHC ligands correlated with the degree of target cell lysis.[62] Later studies have shown that lower affinity pMHC ligands induce less cytotoxic granule polarization in tumor-specific T cells.[63]

In early studies to determine the influence of binding kinetics on potency, Kersch and colleagues generated soluble forms of five known low- or high-potency ligands with similar equilibrium binding affinities (K_D). Their studies indicate that low-potency ligands have faster off-rates than high-potency ligands.[61] However, later experiments demonstrate that immunization with a pMHC that interacts with the TCR longer than the cognate pMHC decreases intracellular signaling, T cell function, and memory

acquisition, suggesting that there is a limit of TCR responsiveness to long-lived interactions.[64]

5.1.4.2 Density of pMHC

The density of pMHC plays a key role in the modulating the T cell-APC interaction and inducing effector function. The effect of cognate pMHC density on mature T lymphocyte activation has been well-studied and characterized.[65-68] It is known that T cells cluster around DCs loaded with higher amounts of antigen than DCs presenting lower amounts of peptide.[69] DCs presenting more antigen form stable interactions with naïve $CD8^+$ T cells, and thus induce proliferation, more quickly than DCs loaded with lower levels of antigen.[69]

Detailed studies indicate that as few as 1-50 pMHC molecules are sufficient to induce a CTL response and trigger target cell lysis; however, it has been demonstrated that a single agonist pMHC on the surface of an APC or bilayer can only induce transient, non-stimulatory calcium flux in $CD4^+$ and $CD8^+$ T cells.[66] In cells with functional co-receptors, sustained calcium mobilization requires the presence of 10 pMHCs on an APC; 20 pMHC complexes are required for sustained calcium response when co-receptors are not present.[70] In more recent studies, Manz and colleagues designed a partitioned bilayer system, whereby the number of agonist peptides per TCR cluster could be modulated, without altering the total number of pMHCs engaged by the cell.[71] Their results indicate that the minimal number of pMHCs needed to trigger calcium signaling is four.[71]

While density plays an important role in T cell activation, it also influences the development of anergy and tolerance. To determine whether naïve T cells maintain tolerance by tuning their TCR signaling to match persistent stimuli, Singh et al., transferred naïve TCR transgenic T cells into mice presenting different levels of cognate

antigen. When transplanted into the recipient containing higher levels of antigen presentation, the adaptively tolerized T cells showed lower levels of TCR signaling, cytokine production, and proliferation compared to T cells residing in the recipient containing lower levels of antigen.[72]

5.1.4.3 pMHC Oligomerization

Encounter of T cells with antigenic stimuli presented by other cells occurs simultaneously with other non-antigen signals such as co-receptor binding, co-stimulation, and interactions with adhesion molecules. Inducing TCR signaling in the absence of APCs can be done non-specifically using phytohemagglutinin (PHA), ionomycin, or agonist TCR/CD3 antibodies; however, stimulating antigen-specific T cell populations requires that the multivalent presentation of agonist pMHC molecules.[73-76]

Initial experiments performed by Abastado et al. have demonstrated that soluble, cognate pMHC Class I monomers are unable to stimulate T cell lines, despite being bound to the cell.[77] However, pMHC dimers sufficiently triggered the cells to secrete IL-2.[74,77] Data presented by Boniface and colleagues has shown that monomeric MCC/IE^k fails to activate $CD4^+$ T cells, even at concentrations 20,000 times higher than the stimulatory doses of MCC/IE^k tetramer.[74] Similar experiments using murine 2C $CD8^+$ T lymphocytes demonstrate that like $CD4^+$ T cells, $CD8^+$ T cells can be stimulated by soluble Class I dimers or higher-order oligomers, but not monomers. Increasing concentrations of soluble MHC monomers can actually compete off the activation observed with oligomeric pMHCs.[75]

The aforementioned studies support the model that dimerization or oligomerization of the TCR-CD3 complex is required for T cell activation. According to Kuhns et al., TCR dimerization via pMHC oligomer engagement polarizes T cells to the

point of APC contact, which in turn collects pMHCs in high local density.[78] Higher pMHC densities might enable more efficient formation of TCR clusters, bringing CD3 ITAMs into close proximity where they are phosphorylated by Src kinases, resulting in sustained signaling.

5.1.4.4 Mechanical Force

The debate over whether dimerization or mechanical force is more important for initiation of TCR signaling is ongoing. The leading theory supporting the requirement of mechanical force in the earliest steps of TCR signaling is based on a conformational change of the TCR-CD3ε complex upon pMHC engagement. In the "classic-style water cooler" model, TCR engagement causes the TCR to pivot on top of and push down the CD3δε and γε heterodimers, while pulling up on the CD3ζζ located on the opposite side of the TCR.[78,79] CD4 and CD8 might contribute to the force by holding the pMHC in an orientation with respect to the TCR and/or cellular membrane.[78,79] The mechanical force of this action may expose tyrosines of the CD3ε and CD3ζζ ITAMS, making them accessible to phosphorylation by kinases.[78,79]

Experiments performed by Kuhns et al. show that destabilizing the TCR-CD3 complex delays the onset and magnitude of intracellular calcium mobilization upon contact of the TCR with agonist pMHC; however, impairment of TCR dimerization only slightly reduces calcium mobilization.[79] The results from numerous experiments that attempt to discriminate between the effects of mechanical force and/or dimerization on early TCR signaling make it hard to clearly determine which mechanism predominates. It is likely that a sum of the two processes plays a role in modulating and sustaining TCR signaling.

5.1.5 Solid-Phase pMHCs Induce TCR Signaling and T Cell Activation

Much focus has been given to the development of cost-effective and efficient methods to stimulate and expand antigen-specific T cells for adoptive transfer. Traditionally, autologous DCs, which express high levels of Class I and Class II molecules, have been used to activate and expand T cells *in vitro*. However, the use of autologous DCs is time-consuming, expensive, and inconsistent due to patient variability. Problems with yield and collection, primary cell expansion, and the inability to implement Good Manufacturing Practice have been driving forces for the development of new methods to stimulate antigen-specific T cells *in vitro*. Several acellular methods employing immobilized pMHC Class I or Class II molecules have been developed and represent "off the shelf", low-cost alternatives to patient-derived DCs.

5.1.5.1 Plate-Bound pMHCs to Expand Antigen-Specific $CD8^+$ T Cells

Peptide-specific $CD8^+$ T cells have been successfully expanded using plate-bound pMHC Class I molecules. In these studies, Schmidt and colleagues immobilized biotinylated cytomegalovirus (CMV) epitope-loaded Class I molecules to plates using a streptavidin linker. PBMCs were seeded on the coated plates and co-stimulated with anti-CD28, IL-2, and IL-7.[80] Tetramer-staining indicated that stimulation with plate-bound CMVp-MHCs resulted in an 80-fold increase in the frequency of CMV-specific $CD8^+$ T cells compared to the un-stimulated cell population.[80] Approximately 80% of the stimulated cells secreted IFNγ during re-stimulation. CMV-specific $CD8^+$ T cells proliferated up to 240-fold after 2 additional stimulations, indicating the promise of using plate-immobilized pMHC molecules for the stimulation and expansion of T cells.[80]

5.1.5.2 HLA-Ig Dimer-Functionalized Microbeads

Microbeads functionalized with pMHC oligomers have shown promise in expanding $CD4^+$ and $CD8^+$ T cells to clinically relevant numbers. In an early study, Oelke et al. designed microbeads capable of providing two signals, antigen engagement (signal 1) and co-stimulation (signal 2), needed for T cell expansion.[81,82] Their aAPC microbeads were functionalized with immobilized HLA.A2-Ig dimers and anti-CD28 antibodies.[81,82] Their studies show that HLA.A2-Ig microbeads, containing HLAs loaded with high-affinity CMVpp65 peptide or low-affinity MART-1 peptide, could induce robust expansion of the respective antigen-specific CTLs.[81,82] Cells stimulated with these beads expanded 10^6-fold after two months and maintained antigen-specificity of greater than 85%.[81,82]

The capacity of aAPCs to generate antigen-specific CTLs was compared to that of autologous peptide-loaded DCs.[81,82] After three rounds of stimulation, MART-1 aAPCs resulted in a higher frequency of tumor-specific CTLs (54.7%) compared to the DC-stimulated population (20.4%).[81,82] 75% of the $CD8^+$ T cells activated with aAPCs were capable of IFNγ secretion and specific lysis of melanoma target cells, indicating maintenance of CTL functionality after several rounds of stimulation.[81,82]

5.1.5.3 HLA Tetramer-Functionalized Microbeads

Maus and colleagues have demonstrated the ability of HLA tetramer- and anti-CD28-functionalized microbeads to expand antigen-specific $CD4^+$ T cells.[83] In their study, Maus et al. tested the efficiency of four types of HLA tetramer-functionalized microbeads to stimulate T cells: a) indirect conjugation of tetramers via anti-Class II monoclonal antibodies (mAbs), b) indirect conjugation of tetramers via anti-streptavidin mAbs, c) direct conjugation of tetramers, or d) direct conjugation of monomers.[83] They found that beads with Influenza-epitope loaded HLA.DR tetramers, indirectly

immobilized via antibody linkers, induced more IL-2 mRNA expression in Influenza-HA-specific $CD4^+$ T cells compared to microbeads in which tetramers or monomers were covalently immobilized.[83] The differences in activation are likely due to the enhanced directionality and flexibility of tetramers immobilized via antibodies, which may improve IS formation.[83]

5.2 OBJECTIVE

The dependence of thymocyte fate on TCR signaling strength poses an interesting question as to whether the strength of signaling can be controlled *in vitro* to direct the development of T cells. It was hypothesized that by engineering substrates with various densities of pMHC ligand, the strength of TCR-pMHC signaling can be controlled, thereby providing an optimal surface for the generation of mature antigen-specific $CD8^+$ SP T cells. In this chapter, the effects of pMHC density and solid phase presentation on TCR signaling and thymocyte differentiation are presented.

Plates and microbeads were fabricated with varying densities of pMHC molecules. The ability of these immobilized pMHC surfaces to stimulate TCR signaling in thymocytes and to induce antigen-specific differentiation was compared to that of soluble tetramers. It was hypothesized that plate- or bead-immobilized pMHC molecules would trigger signaling more efficiently than soluble tetramers, in a density-dependent manner. However, because thymocyte survival is induced by a narrow range of TCR signaling strength, it was uncertain whether higher levels of TCR signaling would directly correlate with more efficient antigen-specific $CD8^+$ T cell differentiation.

5.3 MATERIALS AND METHODS

5.3.1 Microplate Ligand Density Determination

Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates were treated with 200 μg/mL streptavidin (Promega) diluted in phosphate buffered saline (PBS) for 30 minutes at 37°C in 5% CO_2 at 100 μL/well working volume. Solution was removed and wells were washed 2 times with 200 μL/well of PBS. Wells were then blocked with Hank's Balanced Salt Solution (HBSS)/2% bovine serum albumin (BSA) for 45 minutes at 20°C. Blocking solution was removed and wells were washed 2 times with 200 μL/well of PBS. Wells were then incubated with the following concentrations of fluorescein-labeled biotinylated BSA (bBSA-FITC, diluted in PBS): 50, 25, 12.5, 6.25, and 3.13 μg/mL at 100 μL/well working volume. Plates were covered with aluminum foil and incubated at 37°C for 2 hours or at 4°C for 24 hours. Wells were washed 5 times with 100 μL of PBS, and supernatant from washes was saved. The amount of unbound bBSA-FITC was determined by the fluorescence of supernatant, which was quantified using a known standard. Density was calculated by the following equation:

$$\frac{Initial\ Mass\ of\ Ligand - Mass\ of\ Unbound\ Ligand}{Surface\ Area\ of\ Well}$$

5.3.2 Microbead Ligand Density Determination

6 x 10^7 Dynabeads® M-280 Streptavidin (1.0 mg of beads) (LifeTechnologies, 11205D) were aliquoted into low protein-binding microcentrifuge tubes and washed 3 times with 1.0 mL of PBS using a Dynabeads magnet (discontinued, LifeTechnolgies DynaMag™-2 is a new version, 12321D) according to manufacturer's protocols. Beads were then incubated with the following concentrations of bBSA-FITC: 100, 50, 25, 12.5, 6.25, and 3.13 μg/mL at 100 μL/tube working volume (diluted in PBS). Microcentrifuge tubes

were covered with aluminum foil and rotated for at 20°C for 30 minutes. After rotation, beads were washed 3 times with 1.0 mL of PBS using the Dynabeads magnet according to manufacturer's protocols. Supernatant was saved. The amount of unbound bBSA-FITC was determined by the fluorescence of the supernatant, which was quantified using a known standard. Density was calculated using the following equation:

$$\frac{Initial\ Mass\ of\ Ligand - Mass\ of\ Unbound\ Ligand}{Surface\ Area\ of\ Microbeads}$$

5.3.3 MHC Microplate Functionalization

Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates were treated with 200 μg/mL streptavidin (Promega) for 30 minutes at 37°C in 5% CO_2 at 100 μL/well working volume. Solution was removed and wells were washed 2 times with 200 μL/well of PBS. Wells were then blocked with HBSS/2% BSA for 45 minutes at 20°C (room temperature). Blocking solution was removed and wells were washed 2 times with 200 μL/well of PBS. Wells were then incubated with the following concentrations of biotinylated pMHC monomers (Baylor College of Medicine Tetramer Production Facility) containing either ovalbumin (OVA) peptide 257-264 (SIINFEKL) or lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP)34-41 (AVYNFATC) diluted in PBS: 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 μg/mL at 100 μL/well for 2 hours at 37°C. Before plating cells, wells were washed 3 times with 200 μL PBS.

5.3.4 MHC Microbead Functionalization

6 x 10^7 Dynabeads® M-280 Streptavidin (1.0 mg of beads) were aliquoted into low protein-binding microcentrifuge tubes and washed 3 times with 1.0 mL of PBS using a Dynabeads magnet according to manufacturer's protocols. Beads were then incubated

with the following concentrations of biotinylated pMHC monomers containing either OVA peptide 257-264 (SIINFEKL) or LCMV.GP34-41 (AVYNFATC): 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 μg/mL at 100 μL/tube working volume (diluted in PBS). Beads were gently rotated at 20°C for 30 minutes. After rotation, beads were washed 3 times with 1.0 mL of PBS using a Dynabeads magnet according to manufacturer's protocols. Beads were resuspended in 600 μL of PBS (to yield a stock concentration of 10^8 beads/mL) and stored at 4°C for up to 2 weeks.

5.3.5 Thymocyte Isolation

Thymi from C57BL/6 mice or OT-1 transgenic mice (C57BL/6-Tg(TCRaTCRb)1100Mjb/J) (The Jackson Laboratory) were isolated. Thymi were crushed on top of BD Falcon Blue Nylon Mesh 40 μm Cell Strainers (BD Biosciences, 352340) to release thymocytes into a single cell suspension. Red blood cells were lysed using 1X RBC Lysis Buffer (eBioscience, 00-4333-57) at a concentration of 2 mL/thymus for 15 minutes at 20°C (room temperature). Cells were counted and washed with FACS buffer and centrifuged at 300 x g for 5 minutes at 4°C.

5.3.6 Double Positive Thymocyte Sorting

Cells for sorting were resuspended at a concentration of 10^7 cells/mL in PBS containing 0.1% BSA and 2 mM EDTA. Thymocytes were treated with FcR Block (eBioscience, 16-0161) at a 1:100 dilution for 15 minutes on ice, then labeled with FITC-conjugated anti-CD8a (clone 53-6.7, eBioscience, 11-0081) and PECy7-conjugated anti-CD4 (clone RM5-4, eBioscience, 25-0042) at a 1:100 dilution for 30 minutes on ice. Cells were washed with 10-fold excess of PBS containing 0.1% BSA and 2 mM EDTA and centrifuged at 300 x g for 5 minutes at 4°C. Pellets were resuspended at 10^7 cells/mL in PBS containing 0.1% BSA and 2 mM EDTA and transferred to sterile polystyrene 12 x

75 mm test tubes. $CD4^+CD8^+$ DP thymocytes were sorted using BD FACSAria™II (BD Biosciences) and FACSDiva Software (BD Biosciences).

5.3.7 Flow Cytometry

All staining was done in polystyrene 12 x 75 mm test tubes in PBS containing 0.1% BSA and 2 mM EDTA. All tetramers and antibodies were diluted 1:100 and all staining was performed on ice. To determine antigen-specificity, cells labeled with APC-conjugated MHC tetramers for 30 minutes on ice prior to antibody labeling. For all samples, cells were treated with FcR block (eBioscience, 16-0161) for 15 minutes, followed by staining with antibodies. The following reagents were used: APC-conjugated OVAp257-MHC tetramers (SIINFEKL) (Baylor College of Medicine Tetramer Production Facility), APC-conjugated LCMV.GP34-41 MHC tetramer (AVYNFATC) (Baylor College of Medicine Tetramer Production Facility), FITC-conjugated anti-CD8a (clone 53-6.7, eBioscience, 11-0081), PECy7-conjugated anti-CD4 (clone RM5-4, eBioscience, 25-0042), and PE-conjugated anti-CD69 (clone H1.2F3, BD Biosciences, 553237). Analysis was done using BD Accuri™ C6 (BD Biosciences) and FlowJo Version 7.6.5 (Treestar Inc.).

5.3.8 OT-1 Thymocyte Activation using OVA257p-MHC-Functionalized Microplates

OT-1 thymocytes were seeded in pMHC-functionalized Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) at a concentration of 10^5 OT-1 thymocytes at a 200 µL working volume. After 2 hours, cells were collected and processed for flow cytometry.

5.3.9 OT-1 Thymocyte Activation using OVA257p-MHC-Functionalized Microbeads

OT-1 thymocytes were seeded in Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates at a concentration of 10^5 cells/well with microbeads functionalized with varying densities of OVA257p-MHC in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at a 200 μL working volume. For some conditions, bead to cell ratios of 20:1, 10:1, 1:1, and 0.5:1 were used. After 2 hours, cells were collected and processed for flow cytometry.

5.3.10 OT-1 Thymocyte Activation using Soluble OVA257p-MHC Class I Tetramers

OT-1 thymocytes were seeded in Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at a concentration of 10^5 cells/well with soluble OVA257p-MHC tetramers (Baylor College of Medicine Tetramer Production Facility) at a 200 μL working volume. After 2 hours, cells were collected and processed for flow cytometry.

5.3.11 Antigen-Specific Differentiation using LCMV.GP34p-MHC-Functionalized Microplates

Sorted C57BL/6 $CD4^+CD8^+$ DP thymocytes were seeded in Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates functionalized with varying densities of LCMV.GP34p-MHC monomers at a concentration of 10^5 cells/well in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), anti-CD28 (1.0 μg/mL, clone 37.51, eBioscience, 16-0281), anti-CD3 (1.0 μg/mL, clone 17A2, eBioscience, 16-0032), IL-2 (10 ng/mL), and IL-7 (10 ng/mL) at a 200 μL working volume. Cells were cultured for a total of 7 days. Media and supplemental factors were replenished every 3 days. Cells were collected at Day 7 and analyzed for antigen-specificity and T cell markers.

5.3.12 Antigen-Specific Differentiation using LCMV.GP34p- and OVA257p-MHC-Functionalized Microbeads

Sorted C57BL/6 DP thymocytes were in seeded Corning® Not Treated Tissue Culture clear, flat-bottom 96-well plates with OVA257p-MHC- or LCMV.GP34p-MHC-functionalized beads (bead to cell ratio of 10:1) at a concentration of 10^5 cells/well in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), anti-CD28 (1.0 µg/mL, clone 37.51, eBioscience, 16-0281), anti-CD3 (1.0 µg/mL, clone 17A2, eBioscience, 16-0032), IL-2 (10 ng/mL), and IL-7 (10 ng/mL) at a 200 µL working volume. Cells were cultured for a total of 7 days. Media and soluble supplemental factors were replenished every 3 days. Cells were collected at day 7 and analyzed for antigen-specificity and T cell markers.

5.3.13 Statistics

Statistical significance was determined by one-way or two-way ANOVA. Post-hoc multi-comparison analysis was performed using Dunnett's multiple comparison test or Tukey's test. All analysis was performed using GraphPad Prism 6 Software.

5.4 RESULTS

5.4.1 Density on Ligand-Functionalized Microplates

FITC-labeled biotinylated BSA (bBSA-FITC, approximately 66 kDa) was used as a model probe to estimate the ligand density of pMHC (approximately 47 kDa) monomers immobilized on plates. Streptavidin was immobilized on non-tissue culture or tissue culture-treated microplates. Following this, varying concentrations of bBSA-FITC were incubated with the plates at 4°C for 24 hours or at 37°C for 2 hours. Similar ligand densities of bBSA-FITC were immobilized on the plates regardless of plate type, incubation time, or temperature (Figure 5.1).

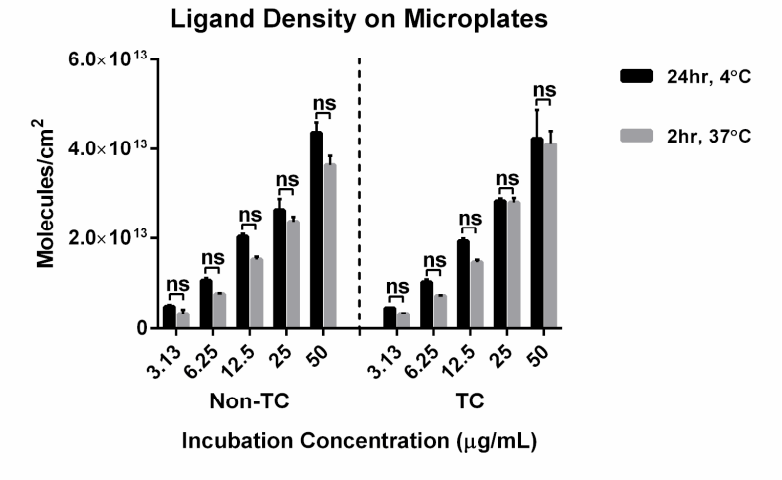

Figure 5.1: Ligand density of bBSA-FITC on polystyrene non-tissue culture treated or tissue culture treated 96-well plates fabricated at 4°C for 24 hours or at 37°C for 2 hours (n = 5, ns = not significant, $P < 0.05$).

5.4.2 Density of Ligand-Functionalized Microbeads

As with the microplate functionalization, bBSA-FITC was used as a model probe to estimate the ligand density of pMHC monomers on stretpavidin Dynabeads®. Varying concentrations of bBSA-FITC were incubated with the beads at 20°C for 30 minutes. As expected, increasing densities of bBSA-FITC were immobilized as the incubation

concentration was increased. However, ligand density (molecules/cm^2) was approximately 10-fold lower on the microbeads compared to the microplate (Figure 5.2).

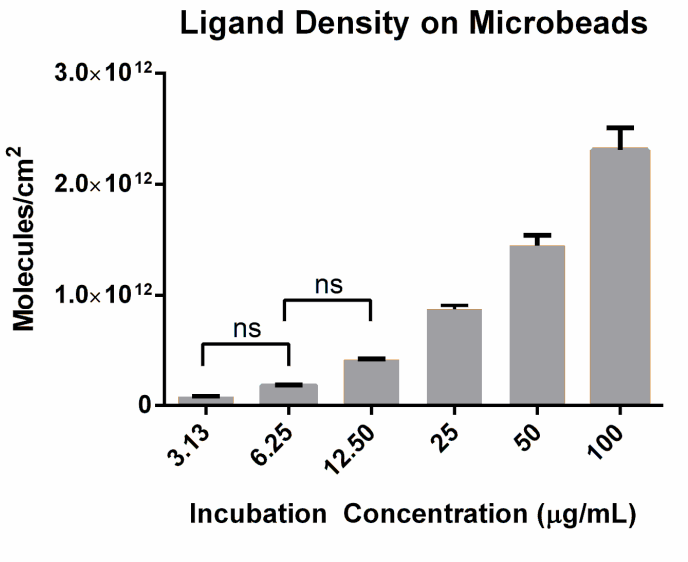

Figure 5.2: Ligand density of bBSA-FITC on magnetic microbeads. Unless indicated, all groups are statistically significant when compared to each other (n = 5, $P < 0.01$).

To compare the effects of surface presentation on TCR signaling and differentiation, and to account for the differences in molecules/cm^2 on plates and beads, equal numbers of molecules pMHC per cell (determined from ligand density quantification) were used for stimulation and differentiation experiments. Density (molecules/cm^2) was varied within each surface (i.e. plates, beads, or tetramers), while simultaneously providing equal numbers of pMHC molecules per cell between groups.

5.4.3 Quantitative Control of TCR Signaling Using pMHC-Functionalized Plates, Microbeads, and Soluble Tetramers

The quantitative effect of soluble or immobilized pMHC molecules on TCR signaling was determined using OT-1 thymocytes. Expression of CD69, an early T cell activation marker, was used to quantify TCR triggering due to agonist OVA257p-MHC

tetramers or OVA257p-MHC-functionalized plates and beads. OT-1 thymocytes were cultured for 2 hours with equivalent numbers of OVA257p-MHC molecules per cell, presented in soluble tetramer, plate-bound or bead-bound form. The data indicates that the percentage of DP cells expressing CD69 increased as a function of ligand density, regardless or presentation method (Figure 5.3A).

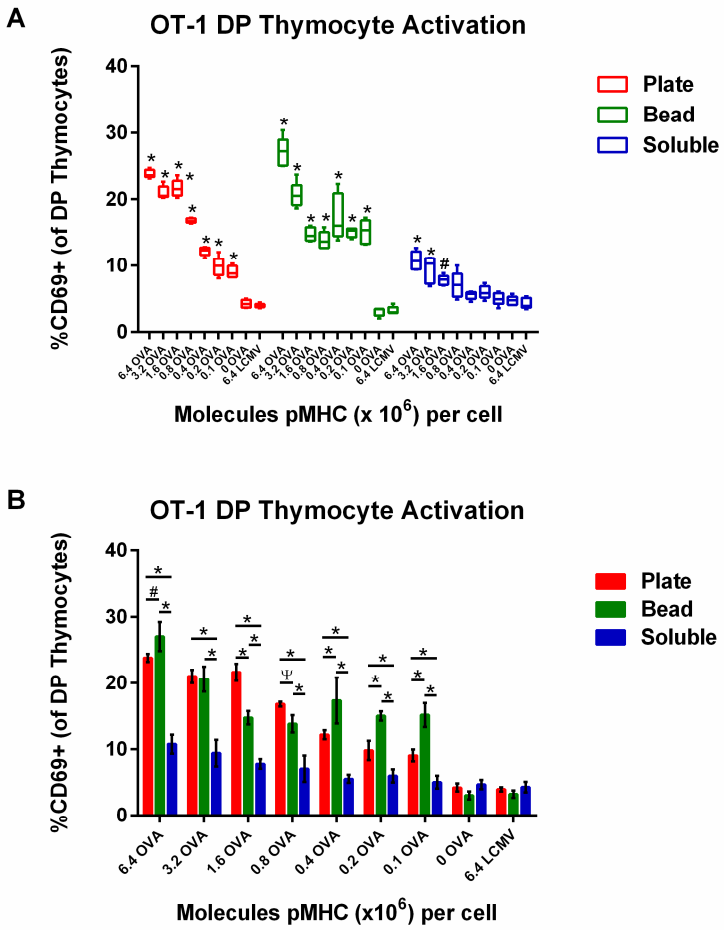

Figure 5.3: Percentage of OT-1 DP thymocytes expressing CD69 after 2 hours of culture with OVA257p-MHC-functionalized plates, microbeads, or soluble tetramers. Effect of A) ligand density (n = 5, $^{*}P < 0.001$, $^{\#}P < 0.05$ when compared to 0 LCMV) and B) immobilization mode (n = 5, $^{*}P < 0.001$, $^{\#}P < 0.001$, $^{\Psi}P < 0.01$) on CD69 expression.

Although equal numbers of pMHC molecules were used to induce signaling, pMHC-functionalized plates and beads stimulated a significantly higher percentage of DP thymocytes compared to the soluble pMHC tetramers (Figure 5.3B). Unlike soluble tetramers, pMHC-functionalized plates and beads induced a significant percentage of DP cells to express CD69 even at the lower densities (0.1 to 0.8 x 10^6 molecules per cell). At the low densities of 0.1 to 0.4 x 10^6 molecules per cell, microbeads activated significantly more DP cells than plates. Interestingly, the percentage of $CD69^+$ cells induced by the pMHC-functionalized beads did not decrease as suddenly it did with the plates, which yielded 2-fold lower numbers of $CD69^+$ cells than the beads at the lowest density tested.

The median fluorescence intensity (MFI) of CD69 expression appeared to follow a density-dependent trend when pMHCs were presented on microbeads and plates, but not soluble tetramers (Figure 5.4A). Soluble tetramers induced significantly lower levels of CD69 in DP cells compared to the plates and beads, at all the ligand densities (Figure 5.4B). Microbeads were capable of inducing higher levels of CD69 expression compared to the plates throughout the range of densities tested. DP thymocytes expressed lower levels of CD69 as plate-immobilized pMHC density decreased. Interestingly, this trend did not occur to the same extent using microbeads, as CD69 expression level remained the same when triggered with bead-immobilized pMHC molecules ranging from 0.1 to 0.8 x 10^6 molecules per cell (Figure 5.4A).

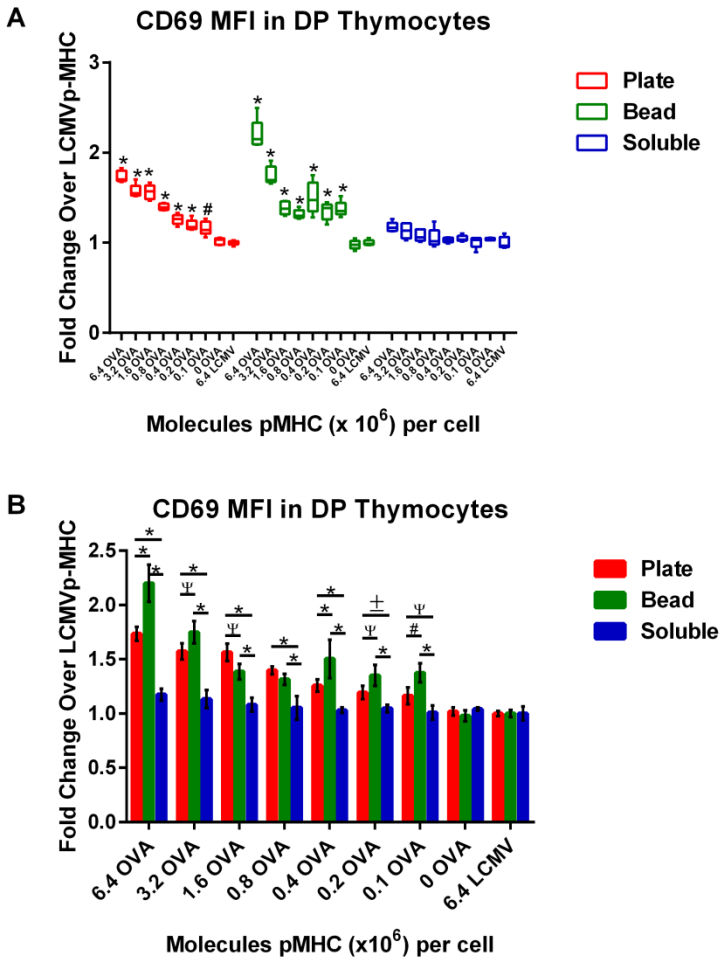

Figure 5.4: Median fluorescence intensity of CD69 in OT-1 DP thymocytes after 2 hours of culture with OVA257p-MHC-functionalized plates, microbeads, or soluble tetramers. Effect of A) ligand density (n = 5,$^{*}P < 0.001$, $^{\#}P < 0.05$ when compared to 0 LCMV) and B) immobilization mode (n = 5, $^{*}P < 0.001$, $^{\#}P < 0.001$, $^{\Psi}P < 0.01$, $^{\pm}P < 0.05$) on CD69 expression.

The effect of varying bead to cell ratios on thymocyte TCR signaling was determined using microbeads immobilized with varying ligand densities of OVA257p-MHC molecules. OT-1 thymocytes were seeded with various OVA257p-MHC-functionalized beads, at bead to cell ratios of 20:1, 10:1, 1:1, and 0.5:1. The results indicate that higher ligand densities (6.4 and 3.2 x 10^5 molecules per bead) and higher

bead to cell ratios (20:1 and 10:1) triggered more DP cells to express CD69 compared to beads at lower bead to cell ratios (Figure 5.5A and 5.5B). At densities lower than 3.2 x 10^5 molecules per bead, increasing the bead to cell ratios did not induce more cells to express CD69.

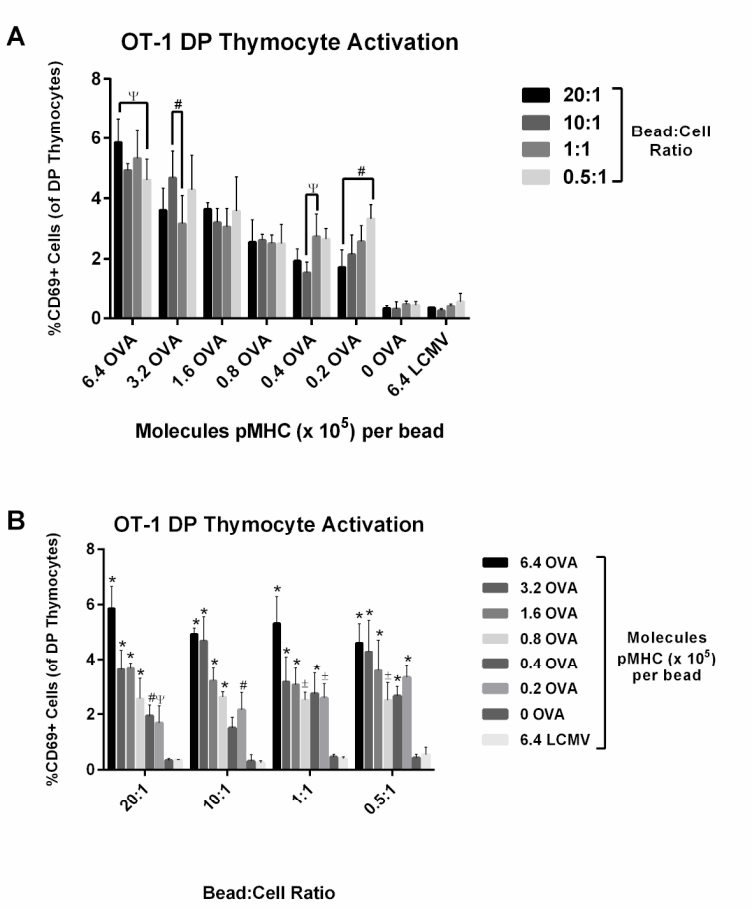

Figure 5.5: Percentage of DP thymocytes expressing CD69 after 2 hours of culture with OVA257p-MHC-functionalized microbeads. Effect of varying A) bead to cell ratios (n = 3, $^{\#}P < 0.005$, $^{\Psi}P < 0.05$) and B) ligand densities (n = 3,*$P <$ 0.0001, $^{\pm}$P < 0.0005 $^{\#}P < 0.005$, $^{\Psi}P < 0.05$ when compared to 0 OVA control) on CD69 expression.

5.4.4 Effect of Ligand Density on Antigen-Specific Differentiation Using pMHC-Functionalized Plates, Microbeads and Soluble Tetramers

The effect of ligand density on antigen-specific differentiation was quantitatively evaluated using OVA257p-MHC- or LCMV.GP34p-MHC-functionalized plates, microbeads, and soluble tetramers. As with the previously described signaling assays, equal numbers of pMHC molecules per cell were used to induce differentiation. LCMV.GP34p-MHC functionalized plates and microbeads were found to be capable of inducing $CD8^+$ LCMV.GP34-specific differentiation.

5.4.4.1 Plate-Immobilized LCMV.GP34p-MHCs Induced LCMV.GP34-Specific $CD8^+$ T Cell Differentiation

Sorted C57BL/6 DP thymocytes were differentiated using streptavidin microplates functionalized with varying densities of biotinylated LCMV.GP34p-MHCs. After 7 days of culture on plates, the cells were collected and analyzed for T cell markers and antigen-specificity. Approximately 50% to 60% of the DP thymocytes differentiated into $CD8^+$ SP T cells (Figure 5.6A). Negligible amounts of $CD4^+$ SP cells were found in all conditions. At the higher ligand densities (6.4, 3.2, and 1.6 x 10^6 molecules LCMV.GP34p-MHC per cell), antigen-specific differentiation was observed. From all of the conditions examined, 3.2 x 10^6 molecules pMHC per cell resulted in the highest percentage of LCMV.GP34-specific cells (approximately 20%) (Figure 5.6B). Similar levels of antigen-specificity were observed using soluble tetramers at 1000-fold higher molecules per cell, suggesting that plate-immobilized pMHC molecules induce differentiation more efficiently than soluble pMHC tetramers (Figure 5.6B). The data indicates an interesting trend between plate-bound pMHC densities, as higher ligand densities resulted in more antigen-specific differentiation.

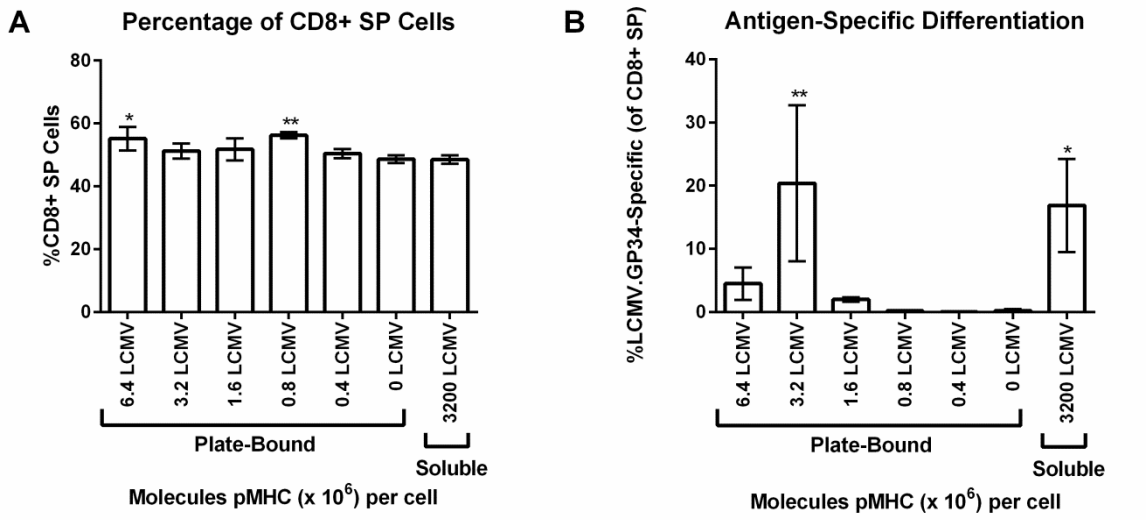

Figure 5.6: A) Percentage of $CD8^+$ SP cells (n = 3, $^{**}P < 0.01$, $^{*}P < 0.05$ when compared to 0 LCMV) and B) LCMV.GP34-specific $CD8^+$ T cells (n = 3, $^{**}P < 0.005$, $^{*}P < 0.05$ when compared to 0 LCMV) after 7 days of culture with varying densities of plate-bound or soluble LCMV.GP34p-MHC molecules.

5.4.4.2 Microbead-Immobilized LCMV.GP34p-MHCs Induced LCMV.GP34-Specific $CD8^+$ T Cell Differentiation

The ability of pMHC microbeads to induce antigen-specific T cell differentiation was determined using streptavidin microbeads fabricated with varying densities of biotinylated LCMV.GP34p-MHCs. In all conditions, regardless of the presence or absence of beads, approximately 50-70% of the C57BL/6 DP thymocytes differentiated into $CD8^+$ SP cells; however, a significantly lower percentage of $CD8^+$ T cells were found in the conditions without beads or containing OVA257p-MHC-functionalized beads (Figure 5.7A). Negligible amounts of $CD4^+$ SP T cells were found in all conditions.

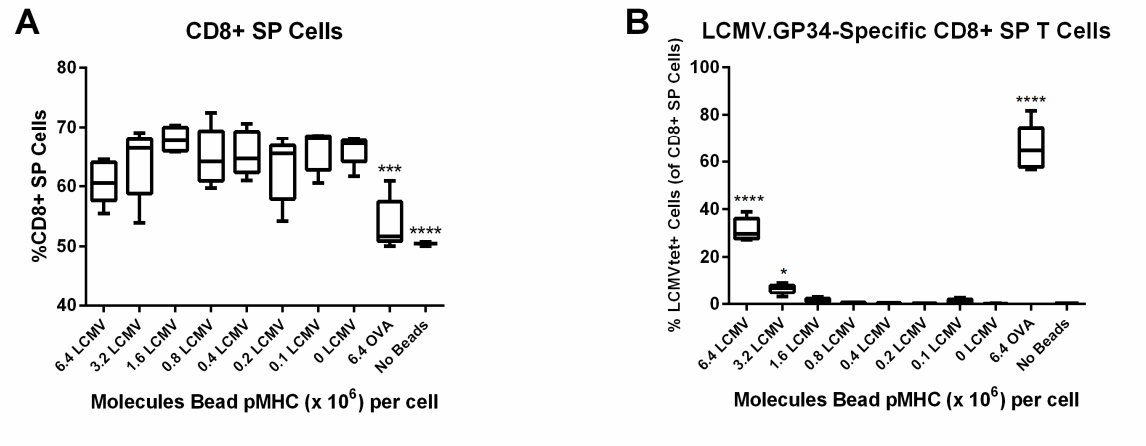

Figure 5.7: A) Percentage of $CD8^+$ SP T cells (n = 5, ****$P < 0.0001$, ***$P < 0.0005$ when compared to 0 LCMV) and B) LCMV.GP34p-MHC-specific $CD8^+$ T cells (n = 5, ****$P < 0.0001$, * $P < 0.05$ when compared to 0 LCMV) after 7 days of culture with varying densities of microbead-bound LCMV.GP34p-MHC molecules.

A density-dependent increase in antigen-specific $CD8^+$ T cell differentiation was observed. Approximately 30% of the $CD8^+$ SP T cells bound to APC-conjugated LCMV.GP34p-MHC Class I tetramers after being trained with beads presenting the highest density (6.4 x 10^6 molecules per cell) of LCMV.GP34p-MHC molecules. Significant LCMV.GP34 antigen-specific differentiation was induced by microbeads presenting 3.2 x 10^6 molecules of LCMV.GP34p-MHC per cell; however, no significant difference in antigen-specificity was generated using microbeads containing 0.1 to 1.6 x 10^6 molecules of LCMV.GP34p-MHC per cell (Figure 5.7B).

Interestingly, the highest amount of LCMV.GP34-specific differentiation occurred when DP thymocytes were cultured with microbeads functionalized with OVA257p-MHC Class I molecules (Figure 5.7B). This observation was consistent with the findings presented in Chapter 4. In the previous chapter, it was shown that DP thymocytes differentiated into LCMV.GP34-specific T cells more efficiently when

trained with OVA257p-MHC tetramers compared to LCMV.GP34p-MHC tetramers. It was hypothesized that the repertoire bias of C57BL/6 mice resulted in deletion of a population of cells in the presence of OVA257p-MHC Class I molecules, thus skewing the surviving cell population specificity. If this hypothesis was correct, bead-presented OVA257p-MHC Class I molecules should induce less OVA257-specific $CD8^+$ T cell differentiation as a function of increasing density.

5.4.4.3 The Effect of Bead-Immobilized OVA257p-MHCs on OVA257-Specific $CD8^+$ T Cell Differentiation

To determine the effect of bead-immobilized OVA257p-MHC molecules on antigen-specific differentiation, streptavidin microbeads were functionalized with varying densities of biotinylated OVA257p-MHCs. C57BL/6 DP thymocytes were sorted and differentiated with the beads for 7 days. After 7 days of culture, the cells were collected and stained for T cell markers and antigen-specificity. CD8 staining shows that approximately 40-60% of the DP thymocytes differentiated in $CD8^+$ SP T cells, regardless of the presence or absence of pMHC-functionalized microbeads. Cells differentiated with the highest density of OVA257p-MHC molecules resulted in significantly less $CD8^+$ T cells when compared to the 0 molecules of pMHC control (Figure 5.8A). This finding was consistent with data presented in Figure 5.7A, where microbeads presenting 6.4 x 10^6 molecules OVA257p-MHC molecules per cell also resulted in significantly lower $CD8^+$ T cells than the 0 molecules of pMHC control.

Staining with fluorescent OVA257p-MHC tetramers shows that OVA257-specificity decreased with increasing OVA257p-MHC density (Figure 5.8B). When 6.4 x 10^6 molecules of OVA257p-MHC molecules per cell were used to induce differentiation, only about 2.0% of the $CD8^+$ T cells were OVA257-specific. This was significantly

lower than background level of OVA257 antigen-specificity generated when using beads presenting 0 pMHCs or no beads (Figure 5.8B).

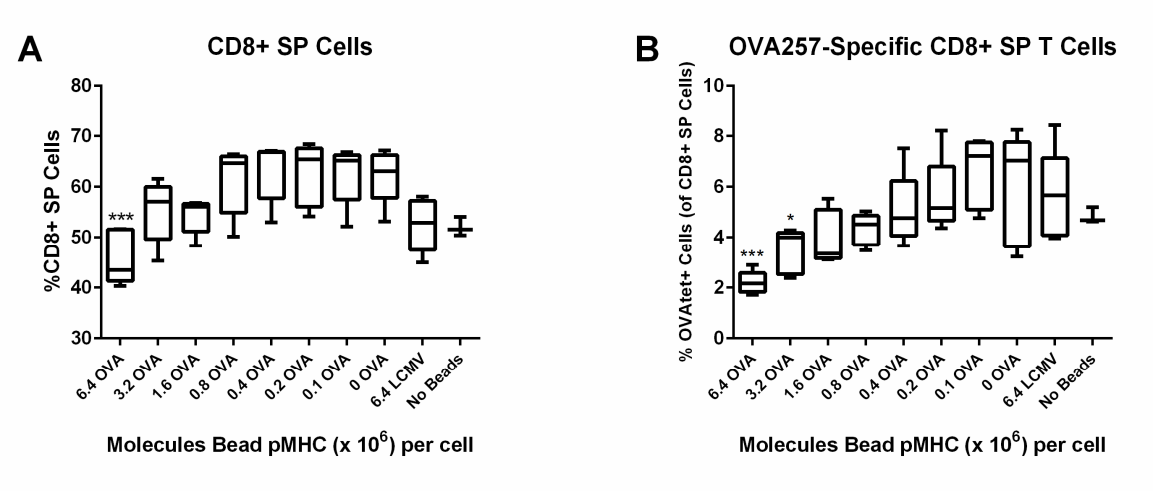

Figure 5.8: A) Percentage of $CD8^+$ SP T cells (n = 5, $***P < 0.0005$ when compared to 0 OVA) and B) OVA257p-MHC-specific $CD8^+$ T cells (n = 5, $***P < 0.001$, $* P < 0.05$ when compared to 0 OVA) after 7 days of culture with varying densities of bead-bound OVA257p-MHC molecules.

5.5 DISCUSSION

The data presented in this chapter indicates that plate- and bead- immobilized pMHC molecules can stimulate thymocytes more efficiently than soluble pMHC tetramers. This suggests that immobilized pMHCs may trigger stronger signaling in thymocytes compared to soluble ligands. The results are consistent with findings from several from other groups, which suggest that immobilized pMHC molecules induce more efficient immunological synapse formation.

Chevrin and colleagues recently demonstrated that CD8 negative T cell hybridomas expressing low-affinity TCRs responded to pMHC Class I complexes immobilized as monomers or tetramers on plastic wells, despite being unresponsive to those pMHCs in soluble monomeric or oligomeric form.[84] Another study performed by Segura et al. indicates that pMHC complexes on the surface of APCs are relatively immobile and cluster around ICAM molecules, and that disrupting the immobility of these complexes impaired antigen-sensitivity and recognition by CTLs.[85] In the plate and bead-based stimulation systems presented, cognate pMHCs, immobilized via densely populated streptavidin linkers, may be present at a local concentration high enough to trigger signaling. The restricted movement of pMHC clusters might facilitate pMHC rebinding to the TCR, allowing engagement of many TCRs in a short frame of time, a mechanism termed signal spreading.[86] According to the results presented here, CD69 expression was induced even at the lowest plate- and bead-immobilized density tested (0.1×10^6 molecules per cell), suggesting that this density was above the critical threshold needed to recapitulate the functions of IS.

When presented at equivalent molecules per cell, bead-immobilized pMHCs were better stimulators of thymocytes than plate-immobilized pMHCs. It is hypothesized that the method of pMHC presentation via microbeads facilitates more TCR-pMHC contacts

per cell. When seeded in a pMHC-coated well, each thymocyte contacts one limited, two-dimensional area of the well once it has settled to the bottom. In comparison, microbeads facilitate multiple three-dimensional thymocyte contacts, as several beads can settle next to, or on top of a cell, enabling the formation of multiple IS'es. Microbeads might also induce signaling more efficiently if they had a higher degree of pMHC oligomerization compared to the plates. It is possible that the plate-adsorbed streptavidin was not fully saturated with biotinylated pMHCs, resulting in disperse distribution of pMHCs that were presented to the thymocyte as lower valency oligomers. Further studies to optimize the streptavidin to pMHC ratio should be performed, but are out of the scope of this work.

LCMV.GP34p-MHC functionalized plates were capable of inducing the generation of LCMV.GP34-specific $CD8^+$ T cells when used at the higher densities tested. Interestingly, plates presenting 3.2×10^6 molecules per cell induced similar levels of antigen-specific differentiation as soluble tetramers presenting 1000-fold more molecules per cell. It was hypothesized that the plate-immobilized pMHC density triggered sufficient signaling in DP thymocytes to induce survival of weakly binding thymocytes. LCMV.GP34-specific T cells were also generated using LCMV.GP34p-MHC-functionalized microbeads. The generation of antigen-specificity appeared to be directly dependent on ligand density, as microbeads presenting more molecules per cell induced more antigen-specific differentiation. The finding that increasing densities of bead-functionalized OVA257p-MHC complexes induced lower amounts of OVA257-specific differentiation may be attributed to the TCR repertoire bias of C57BL/6 mice as described in Chapter 4.

LCMV.GP34p-MHC-functionalized plates and microbeads were capable of inducing antigen-specific $CD8^+$ SP differentiation from T cell progenitors at amounts much lower than what has been previously demonstrated using soluble tetramers. This

interesting finding suggests that pMHC-functionalized plates or microbeads could be used to generate antigen-specific T cells in a more cost-effective manner than soluble ligands or conventionally-used APCs. Microbeads hold particular promise because they can easily be transferred to large bioreactor cultures which are commonly used for scale-up expansion procedures.

5.6 CONCLUSION

In this chapter, the effect of pMHC density and presentation on thymocyte TCR signaling and differentiation was evaluated. It was demonstrated that cognate plate-bound, bead-bound, and soluble pMHC Class I tetramers induced increasing amounts of signaling as a function of ligand density. Interestingly, pMHC-functionalized microbeads stimulated thymocytes most efficiently, possibly due to the unique spatial presentation of pMHCs when immobilized on beads. LCMV.GP34p-MHC-functionalized plates and microbeads were capable of inducing antigen-specific $CD8^+$ SP differentiation from T cell progenitors at lower amounts than what has been previously demonstrated with soluble tetramers, suggesting the potential use of this system for cost-effective and high-throughput antigen-specific T cell generation.

5.7 ABBREVIATIONS

TCR – T cell receptor

pMHC – peptide major histocompatibility complex

pHLA – peptide human leukocyte antigen

HSC – hematopoietic stem cell

APC – antigen-presenting cell

DP – double positive

cTEC – cortical thymic epithelial cell

SP – single positive

TSSP – thymus-specific serine protease

DC – dendritic cell

TRA – tissue-restricted antigen

mTEC – medullary thymic epithelial cell

cDC – conventional dendritic cell

CTL – cytotoxic T lymphocyte

T_{REG} – regulatory T cell

ISP – intermediate single positive

Ig – immunoglobulin

VDJ – variable, diversity, joining

ITAM – immunoreceptor tyrosine-based activation motif

IS – immunological synapse

T_H – helper T cell

$K_{1/2}$ – half-life

K_D – equilibrium binding affinity

PHA – phytohemagglutinnin

aAPC – artificial antigen-presenting cell

CMV – cytomegalovirus

mAb – monoclonal antibody

PBS – phosphate buffered saline

HBSS – Hank's Balanced Salt Solution

BSA – bovine serum albumin

LCMV.GP34p-MHC – lymphocytic choriomeningitis virus glycoprotein epitope (AVYNFATC) pMHC

OVA257p-MHC – ovalbumin epitope (SIINFEKL) pMHC

MFI – median fluorescence intensity

SIINFEKL – (serine – isoleucine – isoleucine – asparagine – phenylalanine – glutamic acid – lysine – leucine)

AVYNFATC – (alanine – valine – tyrosine – asparagine – phenylalanine – alanine – threonine – cysteine)

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Chapter 6: Conclusions and Future Directions

6.1 GENERATION OF ANTIGEN-SPECIFIC CD8$^+$ T CELLS FROM CORD BLOOD CD34$^+$ HEMATOPOIETIC PROGENITORS

6.1.1 Conclusions

In Chapter 3 of this dissertation, a novel differentiation system for generating antigen-specific CD8$^+$ T cells from human cord blood hematopoietic stem cells (HSCs) was successfully demonstrated. This system employed exogenous Notch ligand, Delta like ligand 1 (DLL1), soluble peptide human leukocyte antigen (pHLA) Class I tetramers, and secreted stromal cell factors to induce T lineage commitment and antigen-specific T cell differentiation. Culture of human HSCs with Notch ligands resulted in early T cell differentiation, characterized by CD1a$^+$CD7$^+$ and CD3-expressing CD4$^+$CD8$^+$ double positive (DP) cells.[1]

Addition of cytomegalovirus (CMV) or influenza (GIL) pHLA Class I tetramers induced the differentiation of CMV- and GIL-specific CD8$^+$ single positive (SP) T cells, respectively.[1] Co-culture of CMV tetramer-differentiated effectors with CMV epitope-loaded target cells resulted in more CD107a upregulation compared to co-culture with control target cells.[1] Similar results were seen with GIL tetramer-differentiated cells. Granzyme B activity was assayed to determine cytotoxic killing ability of tetramer-differentiated cells.[1] CMV tetramer-differentiated T cells were of capable of inducing granzyme B activity against CMV epitope-loaded target cells, although with high background activity against control epitope-loaded target cells.[1]

6.1.2 Future Directions

The high background cytoxicity of the stem cell-derived antigen-specific CD8$^+$ T cells suggested that the population needed to be sorted prior to assaying functionality.

This was done using fluorescence activated cell sorting (FACS) and was successfully demonstrated in Chapter 4. It is also possible that the stem cell-derived antigen-specific T cells exhibit cross- or self-reactivity. For clinical use, these cells can be tested for cross- or self-reactivity by co-culture with autologous peripheral blood mononuclear cells (PBMCs). Despite these concerns, the described system presents a novel method to generate antigen-specific T cells without the need for peripheral T cell isolation or the use of genetic modification. This differentiation system has several advantages over the conventional methods used to generate adoptive transfer T cells.

First, cord blood-derived HSCs can be easily retrieved from banks and essentially used as an "on-demand" source for adoptive transfer T cells.[2] The advent of induced pluripotent stem cells (iPSC) have made patient-specific stem cell-derived therapies increasingly possible.[3] iPSCs are somatic cells that have been reprogrammed to a pluripotent state by the transduction with genes known to induce pluripotency in embryonic stem cells (ESCs).[3] These cells have the ability to differentiate into all 3 germ layers, without any chromosomal abnormalities.[3] The use of iPSCs is particularly attractive for clinical purposes because they would allow a patient to donate his or her own somatic cells, without the use of embryos. iPSCs could be used to generate therapeutic cells with patient-specific HLA molecules, eliminating the need to HLA-type and minimizing the chances of developing graft-versus-host-disease (GVHD) associated with allogeneic transfer.

The success of several groups at differentiating ESCs or iPSCs into therapeutic, antigen-specific T cells is promising. The OP9-DL1 co-culture system has been used to direct murine ESCs into functional T cells, which are able to reconstitute the T cell compartment of immunodeficient mice and mount responses against viral infection.[4] Additionally, Lei et al. have shown that murine iPSCs can be differentiated into

progenitor T cells using OP9-DL1 co-culture.[5] They have also shown that murine iPSCs, transduced with T cell receptors (TCRs) recognizing an MHC Class I-restricted ovalbumin (OVA) epitope, can develop into $CD8^+$ cytotoxic T lymphocytes (CTL) when adoptively transferred into recombination activating gene $(RAG)1^{-/-}$ mice.[5] These iPSC-derived cells are functional and respond to peptide stimulation by secreting interleukin (IL)-2 and interferon gamma (IFNγ).[5] In a more recent study, Themeli et al. generated human iPSCs expressing a chimeric antigen receptor (CAR) targeted to CD19.[6] These CAR-transduced iPSCs differentiated into T cells *in vitro*, and could induce tumor regression when transplanted in a mouse xenograft model.[6] These data suggest the potential of the differentiation system, described in Chapter 3, to generate ESC- or iPSC-derived antigen-specific CTLs for therapeutic purposes.

Future studies should move towards replacing the OP9-DL1 conditioned media used for differentiation, with defined, serum-free media that can be reproduced and manufactured consistently. Towards this end, early studies have provided insight into factors that may be needed to induce *in vitro* T cell differentiation. When Delaney et al. used serum-free media supplemented with IL-3, IL-6, thrombopoietin (TPO), FMS-related tyrosine kinase 3 ligand (Flt3L), and stem cell factor (SCF) to expand HSCs, they induced early T cell development in addition to HSC expansion.[7,8] Addition of IL-7 to this cocktail may enhance *in vitro* T cell differentiation.[9-11] Further studies will need to be done to determine if other factors are necessary for *in vitro* differentiation.

6.2 ENRICHMENT AND EXPANSION OF PROGENITOR-DERIVED ANTIGEN-SPECIFIC $CD8^+$ T CELLS

6.2.1 Conclusions

The ability to enrich and expand antigen-specific CTLs is crucial for the generation of sufficient numbers of T cells needed for infusion during adoptive transfer. In Chapter 4, lymphocytic choriomeningitis virus (LCMV).GP34-specific $CD8^+$ T cells, derived from murine progenitors using LCMV.GP34p-MHC Class I tetramers, were sorted by FACS and cultured for 7 days in expansion media. After 7 days, these cells were alive and exhibited specific cytotoxicity, without the high background observed previously. However, the cells did not robustly expand during the 7-day period despite the use of proliferative molecules and cytokines (1.0 μg/mL anti-CD3, 1.0 μg/mL anti-CD28, and 10 ng/mL IL-2), suggesting that the differentiated cells entered a state of T cell dysfunction and indicating that the differentiation or expansion media may need to be optimized.

Interestingly, it was also found that when OVA257p-MHC Class I tetramers were used for differentiation, a higher percentage of LCMV.GP34-specific T cells were generated compared to OVA257-specific T cells. This may due to the inherent repertoire bias of C57BL/6 mice for OVA257-H-$2K^b$ molecules. Since approximately 7% of the DP thymocyte population was found to recognize the OVA257p-MHC tetramer, it was hypothesized that high-avidity cells were deleted when cultured with the OVA257p-MHC tetramers, thus skewing the population. Cells with weak to high avidities for LCMV.GP34-H-$2K^b$ molecules may have been positively selected with the OVA257p-MHC tetramer. When LCMV.GP34p-MHC tetramers were used for differentiation, higher percentages of LCMV.GP34-specific $CD8^+$ T cells were generated compared to OVA257-specific $CD8^+$ T cells, suggesting that the LCMV.GP34p-MHC tetramer

enriched for cells with weak to medium avidity for LCMV.GP34-H-2K^b molecules. The lack of OVA257-specific T cells after culture with LCMV.GP34p-MHC tetramers may have resulted from death-by-neglect. The studies also show that after culture in expansion media, the OVA257-specific CD8$^+$ T cells appear to be cross-reactive and bind to LCMV.GP34p-MHC tetramers.

6.2.2 Future Directions

An in-depth study to determine how varying avidity tetramers induce qualitatively and quantitatively different effects on antigen-specific T cell development could be done using a cohort of OVA-specific tetramers generated from point mutations of the native SIINFEKL epitope. Many of these variants (A2, G4, K4, Q4, R4, T4) are commercially available. Given the speculated bias of C57BL/6 mice for the SIINFEKL pMHC, the use of these variants would hypothetically induce the generation of antigen-specific T cells with varying avidities to the cognate SIINFEKL epitope. These anti-OVA T cells, exhibiting varying avidities for the SIINFEKL tetramers, could be sorted and evaluated for *in vitro* and *in vivo* functionality. The B16-OVA melanoma model could be used to evaluate tumor reduction, survival, and signs of autoimmunity as a function of TCR avidity.

Several possible states of T cell dysfunction were examined in Chapter 4. Due to the maintained functionality of LCMV.GP34-specific CD8$^+$ T cells after 7 days of culture in expansion media, it was hypothesized that the T cells became tolerized, exhausted, or senescent due to the differentiation or expansion culture conditions. To accurately determine the fate of these cells, in-depth cellular and molecular analysis of the differentiated thymocyte population and the antigen-specific T cell population should be

done. It is likely that chronic stimulation by antigen and/or co-stimulatory molecules lead to dysfunction.

Lower concentrations of anti-CD3 and anti-CD28 may need to be used during the differentiation period. While CD28 signaling is required to prevent anergy of mature CTLs, its requirement during development is questionable. Previous studies done by Lin et al. have shown that the presence of anti-CD28 increases the production of antigen-specific T cells, while findings from others suggest that CD28 inhibits SP development *in vivo*.[12-15] In addition, after sorting the antigen-specific T cell population, it is not necessary to include tetramers in the expansion media. Elimination of antigen may prevent dysfunction resulting from chronic stimulation. Future studies should examine varying concentrations and combinations of tetramers and/or co-stimulatory molecules in order to optimize the conditions required to produce T cells that retain the capacity to expand. Additionally, antibody blockade of inhibitory molecules on T cells, or culture of the developing cells with IL-15 or histone deacetylase (HDAC) inhibitors, may be needed to prevent premature exhaustion.[16-19]

6.3 EFFECTS OF MHC LIGAND DENSITY AND PRESENTATION ON TCR SIGNALING AND THYMOCYTE DIFFERENTIATION

6.3.1 Conclusions

In Chapter 5, the effects of pMHC ligand density and solid phase surface presentation on thymocyte TCR signaling and antigen-specific differentiation were examined. In these studies, OVA257p-MHC plates and beads were more efficient at inducing expression of early activation marker, CD69, on OT-1 DP thymocytes compared to soluble tetramers, when given at equal numbers of molecules per cell. From this data, it was also determined that pMHC-functionalized microbeads stimulated thymocytes slightly better than pMHC-functionalized microplates. LCMV.GP34-functionalized microplates were capable of inducing antigen-specific differentiation of DP thymocytes. Interestingly, similar levels of differentiation were seen using 1000-fold higher numbers of pMHC molecules per cell in soluble tetramer form, suggesting that plate-immobilized pMHC oligomers may be more efficient at inducing differentiation than soluble forms. When DP thymocytes were cultured with LCMV.GP34p-MHC-functionalized microbeads, LCMV.GP34-specific $CD8^+$ T cells were generated in a density-dependent manner.

6.3.2 Future Directions

Collectively, the data presented in this dissertation indicates that solid-phase presentation of pMHC molecules can induce TCR signaling in thymocytes more effectively than soluble pMHC tetramers. Antigen-specific $CD8^+$ T cells can be generated using microplates or beads functionalized with much lower amounts of pMHC molecules compared to previous experiments utilizing soluble tetramers. This holds promise for the field of *in vitro* T cell differentiation, as such artificial antigen-presenting cells (aAPCs) could be fabricated in compliance with Good Manufacturing Practice and in a high-

throughput manner. The ability to incorporate bead-based aAPCs into high volume systems, such as bioreactors, holds promise for the development of large-scale *in vitro* T cell differentiation systems.

Future studies should be done to confirm that the antigen-specific $CD8^+$ T cells generated from plate and microbead cultures are functional. Functionality could be evaluated by assaying for granzyme B, using assays similar to those described in previous chapters, as well as assaying for cytokine secretion and proliferation upon antigen stimulation. Further studies to determine if higher densities of microbead-immobilized pMHC molecules can induce more efficient differentiation and to confirm that the observed antigen-specific differentiation is reproducible using other epitopes, would be valuable. Additionally, it was noticed that microbeads aggregated after approximately 24 hours in culture. This aggregation may reduce the amount of ligand surface area available for contact with cells. As a result, it may be necessary to incorporate slight agitation to the cultures to improve bead and cell contact. The detachment of microbeads from differentiating cells should also be optimized. Studies indicate that the majority of microbeads can be removed using magnetic separation following the manufacturer's instructions. However, several rounds of this separation procedure may be needed to maximize cell retrieval, as some cells do end up inside bead aggregates.

6.4 CLOSING REMARKS

In summary, this dissertation presents a unique and novel system from which functional, antigen-specific $CD8^+$ T cells can be generated from human cord blood $CD34^+$ HSCs. To generate clinically relevant numbers T cells, the enrichment and expansion of progenitor-derived antigen-specific $CD8^+$ SP T cells was attempted. Although more work is needed to optimize expansion conditions, the data indicates that cells remain viable and retain specific functionality after enrichment. In addition, the effects of pMHC ligand density and surface presentation on thymocyte signaling and differentiation were evaluated. The results from those experiments indicate that pMHC-functionalized microbeads are capable of stimulating thymocytes and can efficiently induce epitope-specific differentiation, suggesting the potential for large-scale, high-throughput generation of antigen-specific T cells.

6.5 ABBREVIATIONS

HSC – hematopoietic stem cell

DLL1 – Delta like ligand 1

pHLA – peptide human leukocyte antigen

DP – double positive

CMV – cytomegalovirus

GIL – influenza

SP – single positive

FACS – fluorescence activated cell sorting

PBMC – peripheral blood mononuclear cells

iPSC – induced pluripotent stem cell

ESC – embryonic stem cell

GVHD – graft-versus-host-disease

TCR – T cell receptor

OVA – ovalbumin

CTL – cytotoxic T lymphocyte

RAG – recombination activating gene

IL – interleukin

IFNγ – interferon gamma

CAR – chimeric antigen receptor

TPO – thrombopoietin

Flt3L – FMS-related tyrosine kinase 3 Ligand

SCF – stem cell factor

OVA257p-MHC – ovalbumin epitope (SIINFEKL) pMHC

LCMV.GP34p-MHC – lymphocytic choriomeningitis virus glycoprotein epitope (AVYNFATC) pMHC

HDAC – histone deacetylase

aAPC – artificial antigen-presenting cell

SIINFEKL – (serine – isoleucine – isoleucine – asparagine – phenylalanine – glutamic acid – lysine – leucine)

AVYNFATC – (alanine – valine – tyrosine – asparagine – phenylalanine – alanine – threonine – cysteine)

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Glossary

aAPC – artificial antigen-presenting cell

Ab – antibody

ADAM – a disintegrin and metalloproteinase

AIDS – acquired immunodeficiency syndrome

α-MEM – alpha minimum essential media

APC – antigen-presenting cell

APC-conjugated – allophycocyanin-conjugated

APC-labeled – allophycocyanin-labeled

ASK1 – apoptosis signal-regulating kinase 1

ATF2 – activating transcription factor 2

AVYNFATC – (alanine – valine – tyrosine – asparagine – phenylalanine – alanine – threonine – cysteine)

BSA – bovine serum albumin

CAN – calcineurin

CAR – chimeric antigen receptor

CBF1 – C-promoter binding factor 1

cDC – conventional dendritic cell

CEA – carcinoembryonic antigen

CFSE – carboxyfluorescein succinimidyl

CLP – common lymphoid progenitor

CMV – cytomegalovirus

CRS – cytokine release syndrome

cTEC – cortical thymic epithelial cell

CTL – cytotoxic T lymphocyte

CTLA-4 – cytotoxic T lymphocyte associated protein 4

Cy – cyanine

DAG – diacylglycerol

DC – dendritc cell

diAcH3 – diacetylated histone H3

DLL – delta like ligand

DN – double negative

DNMT1 – DNA methyl transferase 1

DP – double positive

DSL – Delta/Serrate/Lag

EBV – Epstein-Bar virus

EDTA – ethylenediaminetetraacetic acid

EGR – early growth respone factor

ES – embryonic stem

ESC – embryonic stem cell

FACS – fluorescence activated cell sorting

FBS – fetal bovine serum

FcR – Fc receptor

FITC – fluorescein isothiocyanate

Flt3L – FMS-related tyrosine kinase 3 ligand

FTOC – fetal thymus organ culture

G-CSF – granulocyte colony-stimulating factor

GIL – influenza

GILGFVFTL – (glycine – isoleucine – leucine – glycine – phenylalanine – valine – phenylalanine – threonine – leucine)

gp – glycoprotein

GVHD – graft-versus-host-disease

GZMM – granzyme M

HAT – histone acetyl transferase

HDAC – histone deacetylase inhibitor

HES – hairy enhancer of split

HLA – human leukocyte antigen

HLA – human leukocyte antigen

HSC – hematopoietic stem cell

hTERT – human telomerase reverse transcriptase

hTrp2 – human tyrosinase-related protein 2

ICAM-1 – intracellular adhesion molecule 1

ICOS – inducible T cell co-stimulator

IFNγ – interferon gamma

IFNγ – interferon gamma

Ig – immunoglobulin

IL - interleukin

iPSC – induced pluripotent stem cell

IRES – internal ribosome entry site

IS – immunological synapse

ISP – intermediate single positive

ITAM – immunoreceptor tyrosine-based activation motif

$K_{1/2}$ – half-life

K32/4-1BB/CD3/28 – K562 erythomyeloid cell line expressing Fcγ receptor, human 4-1BB, coated with anti-CD3 and anti-CD28 antibodies

K_D – equilibrium binding affinity

LAT – linker for activation of T cells

LCMV – lymphocytic choriomeningitis virus

LCMV.GP34p-MHC – lymphocytic choriomeningitis virus glycoprotein epitope (AVYNFATC) pMHC

LDL – low-density lipoprotein

LFA – leukocyte function antigen

LSK – Lin-sca-1+c-Kit+

MAGE – melanoma-associated antigen

MART-1 – melanoma antigen recognized by T cells -1

MDSC – myeloid-derived suppressor cell

MFI – median fluorescence intensity

MHC – major histocompatibility complex

MLR – mixed lymphocyte reaction

MMK – MAP kinase kinase

MP – matrix protein

mTEC – medullary thymic epithelial cell

NFAT – nuclear factor of activated T cells

NF-κB – nuclear factor-κB

NK – natural killer

NLVPMVATV – (asparagine – leucine – valine – proline – methionine – valine – alanine – threonine – valine)

OP9 – bone marrow derived stromal cell

OP9-DL1 – bone marrow derived stromal cell expressing delta like 1

OP9-DL4 – bone marrow derived stromal cell expressing delta like 4

OT-1 – transgenic mouse line with T cells expressing receptors specific for OVA257p-MHC

OVA – ovalbumin

OVA257p-MHC – ovalbumin epitope (SIINFEKL) pMHC

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PE – phycoerythrin

PECy7 – phycoerythrin cyanine 7

PHA – phytohemagglutinnin

pHLA – peptide human leukocyte antigen

PIP3 – phosphatidylinositol-(3,4,5)-trisphosphate

PKCα – protein kinase Cα

PLCγ – phospholipase Cγ

pMHC – peptide major histocompatibility complex

PRF1 – perforin 1

PTLD – post-transplant lymphoproliferative disease

RAG – recombination activating gene

REP – Rapid Expansion Protocol

RTOC – reaggregate thymus organ culture

SCF – stem cell factor

scFv – single chain variable fragment

SCID – severe combined immunodeficiency

SIINFEKL – (serine – isoleucine – isoleucine – asparagine – phenylalanine – glutamic acid – lysine – leucine)

SLP76 – SH2 domain-containing leukocyte protein of 76 kDa

SP – single positive

TAA – tumor-associated antigen

TAP1/2 – transporter associated antigen presentation 1/2

T_{CM} – central memory T cell

TCR – T cell receptor

T_{EM} – effector memory T cell

T_H – helper T cell

TIL – tumor-infiltrating lymphocyte

T_N – naïve T cell

TNFα – tumor necrosis factor alpha

TNFR – tumor necrosis factor receptor

TPO – thrombopoietin

TRA – tissue-restricted antigen

T_{REG} – regulatory T cell

TRUCK – T cell redirected for universal cytokine-mediated killing

TSP – thymus seeding prognitor

TSSP – thymus specific serine protease

UCB – umbilical cord blood

VDJ – variable, diversity, joining

VPA – valproic acid

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Vita

Tracy P. Ooi was born in Plantation, Florida, where she spent her childhood with her parents and two younger sisters. She graduated as salutatorian from Nova High School in 2005 and subsequently enrolled at Northwestern University, where graduated with Senior Honors with a Bachelor of Arts in Biological Sciences and a concentration in Physiology in 2009. During college, Tracy was an NSEC REU Fellow and received the Summer University Research Grant and the Weinberg College of Arts and Sciences Research Grant. She went on to pursue a Ph.D. in Cellular and Molecular Biology at The University of Texas at Austin, where she conducted research in the field of T cell immunoengineering and immunotherapy under the supervision of Dr. Krishnendu Roy and Dr. Haley Tucker. Her work in the field resulted in one first-author publication and over 10 conference proceedings. In June 2013, Tracy received a RISE Professional Fellowship from the DAAD to fund a summer research internship at the Paul-Ehrlich-Institüt in Langen, Germany, where she worked under the supervision of Dr. Jörg Kirberg, investigating the mechanisms regulating peripheral T cell homeostasis. During graduate school, Tracy was also an active member in the American Association of Pharmaceutical Scientists and helped found the Biotec Section Student Committee, where she served as Chair and Student Representative from 2012 to 2014.

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